

ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES
FROM *EUPATORIUM SEROTINUM*

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
requirements for the degree of Master of Science in Chemistry.

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LIST OF ABBREVIATIONS

^{13}C = Carbon 13, used for NMR spectroscopy
aq = aqueous
 CH_2Cl_2 = dichloromethane
COSY = Correlation Spectroscopy
ESI-MS = Electrospray Ionization Mass Spectrometry
g = gram
HMBC = Heteronuclear Multiple Bond Correlation Spectroscopy
HPLC = High Performance Liquid Chromatography
HSQC = Heteronuclear Single Quantum Coherence
 m/z = mass-to-charge ratio
MeOH = Methanol
mg = milligrams
nm = nanometer
NMR = Nuclear Magnetic Resonance
Ppm = parts per million
 δC = chemical shift of carbon NMR reported in parts per million
 δH = chemical shift of proton NMR reported in parts per million
 μM = micromolar
 $\mu\text{g/mL}$ = micrograms per milliliter

ABSTRACT

ISOLATION AND CHARACTERIZATION OF SECONDARY METABOLITES
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Western Carolina University (March 2012)

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A phytochemical investigation into the species *Eupatorium serotinum* (*Boneset*), which is native to the mountains of western North Carolina, was conducted. The isolation of three secondary metabolites from *Eupatorium serotinum* was accomplished by a modified Kupchan method and various chromatographic separations. Various standard and correlation NMR techniques were used to determine the identity of these compounds. The compounds discovered were 4-aromadendren-3-one, 7 δ -methoxy-4(14)-oppositen-1 β -ol, and germacra-4(15),5,10(14)-trien-1 β -ol. These compounds were newly discovered in *Eupatorium serotinum*, but all have been found in other species before. Two of these compounds have been found in various members of the aster family, of which *Eupatorium serotinum* is from, and may have use as biomarkers.

1 INTRODUCTION

1.1 Natural Products

1.1.1 Introduction To Natural Products

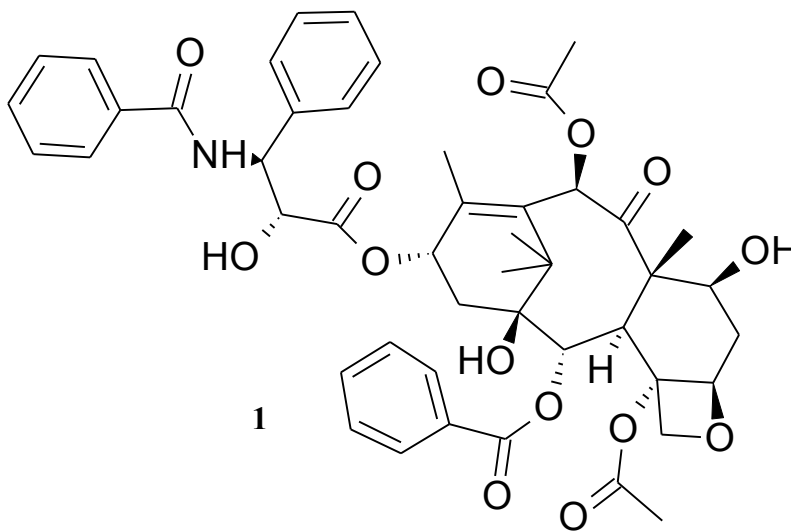
Since the dawn of civilization, people have relied on herbs and other folk remedies to help cure their sick and injured. What these early humans did not know, however, was how chemically complex the plants they had been using were. The fact that the plants worked and helped alleviate some of their problems was attributed to spirits and magic. In many cases scientists have now determined that the plants they used work due to one or several chemical compounds contained within the plant. These compounds interact with the human body in such a way as to help treat whatever sickness or condition for which they were prescribed. The most widely known example being the white willow tree, which was used by several ancient cultures including the ancient Egyptians¹ and the Greeks² to treat aches and pains, turned out to contain salicin, which is the naturally occurring form of salicylic acid. Salicylic acid is the precursor to acetylsalicylic acid, otherwise known as aspirin.

Since originally all medicines were of natural origin, it is also the earliest form of medicinal chemistry. Those doctors, midwives, and medicine men might not have realized it, but the tinctures, tonics, and teas they made were the initial steps on the journey that has created modern medicine today. Some of the most popular medicines of today are the outcome of natural products research.

Natural products chemistry is the study of chemical compounds that are created by or found in living organisms. Natural products chemists primarily focus on secondary metabolites. Secondary metabolites are the compounds that are not produced in large amounts in all organisms, but are of limited distribution. Secondary metabolites are unique to a genus or species and in most cases the natural functions of these compounds are not well known.³ Common types of secondary metabolites are toxins for defending against predators, pheromones for signaling and mating purposes, and coloring agents just to name a few.

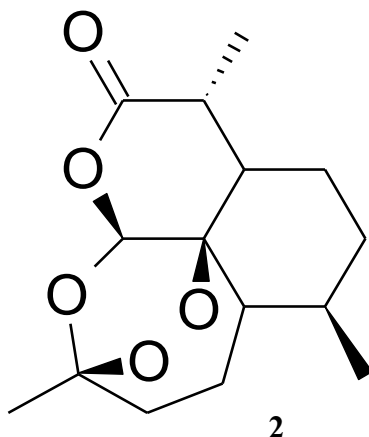
1.1.2 Taxol

One of the most widely known examples of a drug discovered by natural products research is Taxol® or paclitaxel (the generic name) (**1**), a widely used anticancer drug that was discovered from the bark of the pacific yew tree (*Taxus brevifolia*).⁴ Aside from Taxol's use in fighting ovarian, breast, and several types of lung cancer, it has also been used as a molecular scaffold for developing several synthetic derivatives that are more effective for their selected cancerous target.⁵



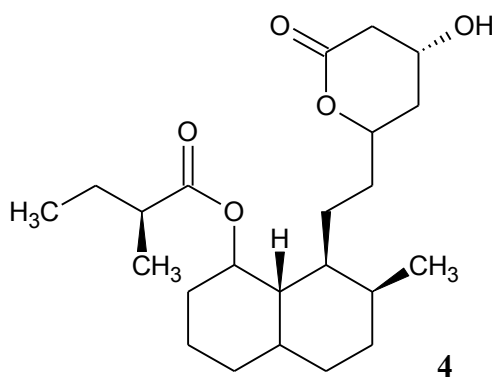
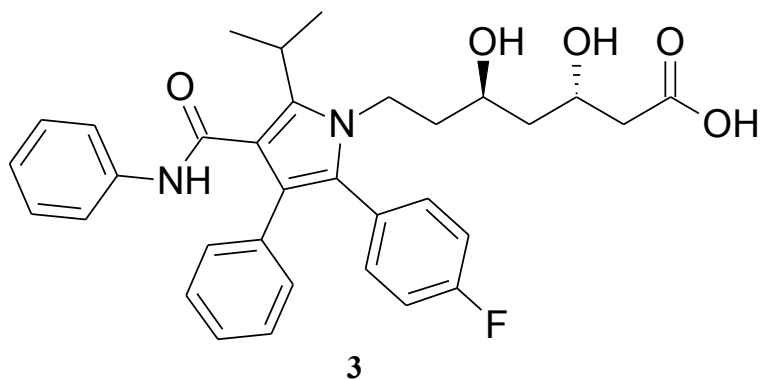
1.1.3 Artemisinin

Another example of an excellent medicine from natural products is artemisinin. Artemisinin (**2**) is an anti malarial compound that was isolated from sweet wormwood or qinghao in traditional Chinese medicine (*Artemisia annua*).⁶ It is now used the world over as a treatment for malaria.



1.1.4 Atorvastatin

A good example of a natural product derivative, atorvastatin, is one of the best selling drugs on the prescription market, selling over 11 billion dollars worth in 2004.⁷ Atorvastatin (**3**) is based on mevastatin (**4**), which is produced by cultures of *Penicillium citrinum* and *Penicillium breviocompactum*.⁸ Most compounds based on mevastatin are used in the treatment of cardiovascular disease. Aside from these examples there are plenty of other natural products and derivatives of natural products in the medicinal field today. Approximately 50% of all small molecule compounds approved for medicinal use are a result of research related to natural products.⁹



1.1.5 Phytochemistry

There are several fields of natural product chemistry, mostly divided up by the types of living organisms being examined. Phytochemistry is the natural product field devoted to the study of chemicals from plants. It is one of the oldest natural products fields and has provided many useful compounds. Another type of investigation is bacterial and fungal culture examinations which have been widely practiced since the 1930s, investigations in marine organisms have also gained a lot of popularity recently.

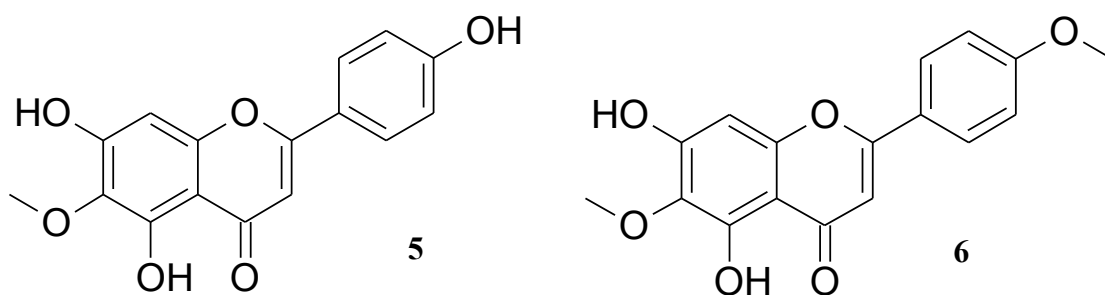
The identification of compounds from plant extracts is useful as it helps lay the foundation for the study of the medicinal value of the plant species. If a specific secondary metabolite is found in several plants that have the same or similar medicinal effects, then that compound might be the biologically active compound that causes the effect. Secondary metabolites that are found predominately in species of a particular genus or family can be used as a biomarker. The presence of the biomarker can help lead to the identification of unknown species and samples. Biomarkers can also be used to help identify environmental and biological factors aside from taxonomy. If an isolated secondary metabolite is similar to a known chemical that has similar effects as the plant extract, then that information can be used to study how both of those compounds work. The phytochemical analysis of plant extracts from medicinal plants is the foundation for building an understanding of not only how and why the selected plants produce the desired medicinal effect, but also in understanding how similar plants of different species might be used medicinally as well.

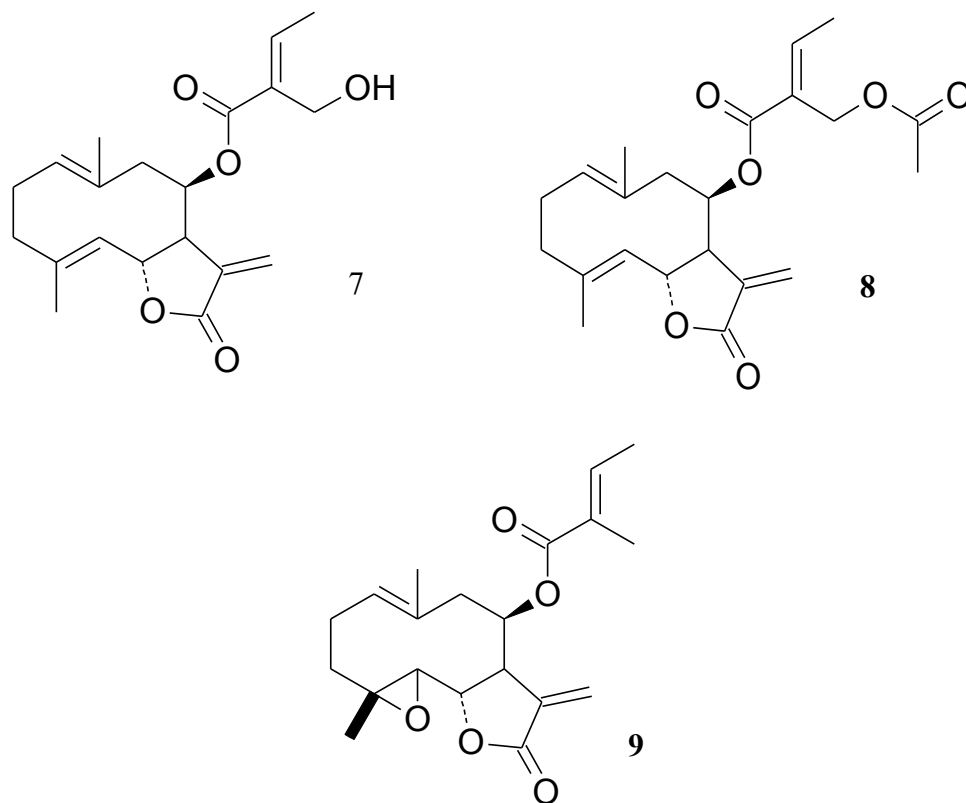
1.2 Boneset (*Eupatorium serotinum*)

The plant selected for this phytochemical analysis is Boneset (*Eupatorium serotinum*). Boneset is native to the southern Appalachian mountains. Other common names for *Eupatorium serotinum* are Late Boneset or Late Thoroughwort. Boneset has had little study done on its secondary metabolite

composition. This plant is of interest because Boneset has some documented use as a medicinal plant by the early settlers and the Native Americans of the Appalachia region as well as in other regions of the United States. As referenced by its earlier name of Feverwort, Boneset was primarily used for reducing fevers.¹⁰ Boneset is thought to be a diaphoretic and helps to break fevers by causing perspiration. Boneset was also used for treating aches and pains, especially those caused by arthritis, as well as used with peppermint to make a tea that is helpful for treating coughs.

Boneset is a member of the aster family (Asteraceae)¹¹ and generally grows to a height of one meter with a maximum height of about two meters with white hairs on its stem. The leaves are generally ten centimeters wide with a width of 15 or more centimeters and have 5 veins and toothed edges. The flowers are small and are clustered into groups of around 15 resembling a larger flower and blooms from August to October. Boneset's common habitats are prairies, plains, meadows, pastures, savannahs, and woodland edges.¹² Some phytochemical work has been performed on *Eupatorium serotinum* and several sesquiterpene lactones have been reported.¹³ The compounds reported are germacranolides (**7,8,9**) as well as hispidulin (**5**) and pectolinarigenin (**6**).





1.3 Overview Of Methods

1.3.1 Liquid Chromatography

The primary method of compound purification to be used is chromatography. While chromatography comes in many forms, the basics are always the same. In the phytochemical analysis of the target plant liquid chromatography will be used primarily due to its availability, the simplicity of collecting samples from this technique, and the fact that it will likely be effective for all compounds in the extract.

Liquid chromatography involves the interaction of two separate components, the mobile phase and the stationary phase. The mobile phase is a

solvent or mixture of solvents that will dissolve the sample to be separated. The stationary phase is immiscible in the mobile phase and is chosen for its affinity for the compounds found in the sample. The mobile phase moves through the stationary phase and the interactions of the sample compounds with the stationary phase are what will cause the separation to occur. The higher the affinity for the stationary phase a compound has, the longer it will take that compound to travel through the column. As the mobile phase flows through the column, the separation occurs due to the differences between the interaction with the stationary phase and the individual compounds. Elution is when chemical compounds are moved off the column by the mobile phase. The mobile phase is sometimes called the eluent and the compounds themselves are called the eluate.

The stationary phase is contained in some way, generally either in a column of some kind or in a planar system.¹⁴ Column chromatography is the most common method for larger scale separations and is also the type of chromatography used in many instruments. Planar chromatography is generally used for small-scale separations and as a method of analysis. Both planar and column chromatography have the same kind of interactions, so provided the mobile and stationary phases are the same, the results are very similar. Due to this, Thin Layer Chromatography (TLC), a type of planar chromatography, is used to test the effectiveness of a mobile or stationary phase with a small part of the sample to be separated in order to maximize effectiveness on the large scale. TLC is also used to analyze the eluent and eluate collected during traditional

column chromatography. The eluent is collected in batches called fractions. These fractions are analyzed by TLC and the fractions that contain the same compound or mixture of compounds are combined.

There are two different types of column chromatography, but while they both follow the same principles, they are different in how they are implemented. Open column chromatography is a commonly used method in organic labs because it can be used on almost any scale with efficiency. Open column chromatography consists of a chromatography column that is filled with the appropriate stationary phase. Generally, a column is filled by pouring a slurry that consists of the stationary phase in an appropriate solvent. In the case of a mobile phase that consists of a mixture of solvents, the slurry will use the solvent that has the best affinity for the stationary phase. This allows the stationary phase to settle, prevents gas bubbles, and promotes a uniform stationary phase. The solvent is allowed to flow through the column until it is level with the stationary phase. Then additional mobile phase is added and flows through the column in order to create a uniform environment within the stationary phase, which is ideal for chromatography. Once the mobile phase has equilibrated with the stationary phase and is level with the top of the stationary phase again, the sample is loaded onto the column and is adsorbed into the top of the stationary phase. Mobile phase is added and then the separation begins.

Open column chromatography is such a widely used technique because it only requires a glass column with a stopcock or valve on one end, and the selected stationary and mobile phases. A common addition to this type of

chromatography, called flash chromatography, is some kind of pressure applied at the top of the column. The most common type is air pressure usually from a pressurized air source or a hand pump. This is added in order to speed up the chromatography process.

1.3.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is the other type of column chromatography. It is abbreviated HPLC and is sometimes referred to as high pressure liquid chromatography. HPLC has a permanently packed column as opposed to open column chromatography, and the column is generally made out of an unreactive metal. The reason for this is that the mobile phase is pushed through the stationary phase at high pressures. The typical pressures used in HPLC range from 500 to 4000 psi.¹⁵ Because of the high pressure required, all HPLC's have a pumping system that is integral to the instruments operation. Most modern HPLCs also have a solvent reservoir and a mixing chamber, which allows the user to control the volume ratio of the mobile phase solvents through the instrument interface. The pumping system needs to be of a high enough quality that it can consistently produce a high pressure and relatively pulse-free output. Relatively pulse free means that the mobile phase is moving at a constant pressure and flow rate throughout the entire operational period.¹⁶ This is a requirement because the variation in pressure causes the efficiency of the separation to go down. A completely pulse free pump would be ideal but due to

the mechanical action employed in high pressure pumps pressure pulses are unavoidable. This difference, however, is almost inconsequential in most HPLC applications.

Requirements for the solvents used in HPLC are filtering and degassing. Filtering the solvents will get rid of particulates that might contaminate the sample and can block the flow through the HPLC column. Particulates that are large enough can cause permanent damage to the column. Degassing helps to remove dissolved gases that are in the solvent. This is accomplished by pulling a vacuum on the solvent container while vibrating or sonicating. Both the particulates and the dissolved gas bubbles can also cause the efficiency of the separation to go down. Dissolved gas bubbles reduce separation efficiency by causing band broadening and interfering with some detection systems.¹⁷

While the most common uses of HPLC are for small amounts of material, there are preparative HPLC columns that are designed for the purification of large samples. Another feature that most HPLC's have is the ability to perform chromatography under gradient conditions. An isocratic method is a method where the solvent system is the same throughout the entire run. An example would be a run where the solvent system is constantly 70% methanol. A gradient method is one where the solvent is not the same throughout the whole run. An example of this would be where the solvent composition starts off being 50% methanol and over the course of the 30-minute run it increases to 100% methanol. The best HPLC's have the capacity to execute a run where there can

be several sections of isocratic and gradient solvent conditions. Fine tuning of these methods can greatly affect the separation performance.

Aside from the column, pump, and solvent systems, all HPLC systems have a detector of some kind. The detector is necessary for analyzing the eluted material. In preparative HPLC the detector is used to determine when to separate the fractions in order to purify compounds. Quantitative HPLC uses the detector to measure the amount of material eluted. The most common type of detector is a UV-Vis absorption detector. These detectors measure the amount of ultraviolet and visible light absorbed by the analyte components as they elute. While variable wavelength detectors and photodiode arrays are capable of measuring more than one wavelength, a commonly analyzed wavelength is 254nm. Absorption is measured at 254nm because compounds with substituted aromatic chromophores usually absorb around this wavelength.¹⁸ Setting the detector at this wavelength ensures that the majority of the compounds being analyzed will show absorption while avoiding interferences caused by solvents. Aside from the UV absorption detectors, most HPLC systems can be interfaced with a variety of detectors ranging from the simplest such as refractive index detectors to being routed into another instrument such as a mass spectrometer. After flowing through the detector, the eluent can be collected and separated according to the chromatogram. This separation is the main goal of preparative HPLC.

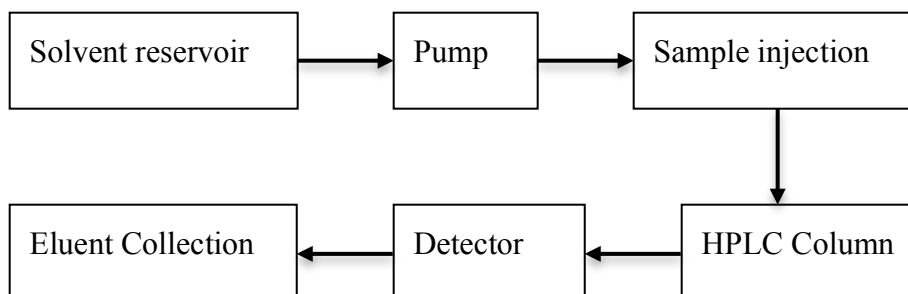


Figure 1.1: Block diagram of a typical HPLC instrument

1.3.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

The primary method of identifying isolated compounds, as well as a guiding force in prioritizing which fractions should be examined, is nuclear magnetic resonance spectroscopy (NMR). NMR at its very core is based upon the interactions between individual nuclei, their neighboring atoms, and the magnetic field in which NMR takes place. NMR works because the magnetic spin of the individual nuclei will align when placed inside a larger electromagnetic field, which is generated by either a large magnet or superconductor solenoids that generate an electromagnetic field. The typical NMR today is a Fourier transform NMR, which uses a pulse of radio frequency energy to excite the spins of the sample's nuclei into a high energy state. As they relax into a lower energy state, the excited nuclei precesses¹⁹, which means it is rotating around its aligned axis. This is detected by a change in the radio frequency field around them. The change in the field is recorded by a receiver coil perpendicular to the main magnetic field.²⁰ This receiver coil is generally the same one used to pulse the sample. The signal from the receiver coil is then digitized and stored on the

computer attached to the instrument, which performs the Fourier transform process on the raw data and generates the spectra.

1.3.4 DEPT

Several types of NMR spectroscopy are used in order to generate spectra that when combined will allow for the structural determination of the sample compound. Both ^1H hydrogen, the most common isotope analyzed by NMR, and ^{13}C carbon analyses are performed. In addition to the standard hydrogen and carbon data that is usually collected, a DEPT experiment is very informative. DEPT stands for distortionless enhancement by polarization transfer²¹ and is usually followed by a number, generally 135 or 90. The number is for the proton pulse angle that is set for the DEPT experiment. The data obtained from the DEPT-135 experiment looks similar to a standard ^{13}C NMR experiment but with a few key differences. The major difference is that some of the carbon peaks are pointed down instead of up. This is because during the DEPT experiment the electromagnetic pulses are delayed in a certain way as to phase the different types of carbons either up or down. In a DEPT-135 experiment the methylene carbons are phased down and the methine and methyl carbons are phased up. So any peak on the DEPT-135 data that is pointed down is a CH_2 carbon and the ones pointing up are either CH or CH_3 carbons. Quaternary carbons, or carbons without any hydrogen attached do not show up in the DEPT-135 experiment. A DEPT-90 experiment will only show methine, or CH, carbons. Upon comparing

the DEPT data to a standard ^{13}C carbon experiment data the multiplicity of each carbon peak can be assigned. This information is very useful in determining the structure of a target compound.

1.3.5 Correlation Spectroscopy

In addition to the hydrogen and various carbon one dimensional NMR experiments, several other two dimensional correlation techniques are performed as well. COSY or ^1H - ^1H correlation spectroscopy can determine the relationship between different nuclei within the sample molecule, and generates a two dimensional spectrum that shows which hydrogen atoms are coupling with each other. COSY is performed by using a series of pulses designed to make the spins of the nuclei interact with each other. The receiver coil records the changes in the magnetic field caused by the interactions. COSY is the form of correlation NMR where the interactions between hydrogen nuclei are recorded. COSY gives important information through these proton couplings that is very useful in structure elucidation.²² The data is collected and undergoes a double Fourier transformation which gives a spectra with two axes that are chemical shift measurements. The resulting data is a contour plot, and the peaks formed by the contour plot are used to determine which protons are coupled. There are similar C-H and C-C Correlation NMR methods which are used to determine the correlations between the specified nuclei. Two types of C-H Correlation NMR are commonly used; heteronuclear single quantum coherence (HSQC) and

heteronuclear multiple bond coherence (HMBC). HSQC gives data about proton nuclei that are directly bonded to the specified heteroatom, usually carbon. HMBC gives data about proton nuclei that have a long-range correlation to the specified heteroatom. The distance between the proton and carbon is usually two or three bonds.²³ All of these methods help us determine where the specific nuclei are in relation to the rest of the molecule. The data gathered in these two dimensional correlation spectroscopy experiments are a major source for information regarding the substructure of the sample compound. HSQC data is used to correlate protons to the carbons they are bonded to, and is especially useful in conjunction with DEPT-135 in determining how many protons are bonded to each carbon atom. The HMBC data can then be used to determine which carbon atoms are near one another in the compound's structure. By using all of the NMR data available a section of the substructure of the molecule or the complete structure of the molecule can be elucidated. If only the substructure can be identified then it can be used to help determine the identity of the target compound.

1.3.6 Mass Spectrometry

Another method of identifying and verifying the structure of our isolated compounds is mass spectrometry. Mass spectrometry is not usually a primary method of structure determination for natural products. With the correct ionization method, such as electrospray, it can be used to verify the molecular mass of a

molecule. The data obtained from mass spectroscopy is in the form of mass to charge ratio of ions or ionic fragments from the sample compound. This data can be used to determine substructures of the sample molecule and the combination of the fragments can be used to determine the identity of the sample compound.

Mass spectrometry is accomplished by a basic method. First the compound is ionized. The ions are then sorted by their mass to charge ratio and this mass to charge ratio is recorded by the detector. How these steps are accomplished varies depending on what kind of instrument is used. The type of mass spectrometer to be used in the current project is an electrospray ionization mass spectrometer with an ion trap. The electrospray ionizer uses a needle held at high voltage through which the sample solution is sprayed out. The solution is charged by the voltage difference, and as the droplets come out they are ionized. A drying gas is moved over the droplets of sample solution and helps remove solvent. The ionized droplets are then attracted to the entrance of the mass analyzer.²⁴ Finally, the droplets reach a point where surface tension is overcome by the saturation of sample and burst, atomizing the solvent and leaving the charged sample ions to flow into the mass analyzer.

The type of mass analyzer in the mass spectrometer to be used is a quadrupole ion trap. The quadrupole mass analyzer refers to how the ions are separated by their mass to charge ratio. A quadrupole mass analyzer contains a mass filter which consists of four charged poles with a DC voltage and an alternating radio frequency voltage.²⁵ The frequency of the voltage on the poles is changed so as to select for a specific mass to charge ratio of the ions which

pass through it.²⁶ Once the ions are through the quadrupole mass analyzer, they are moved to the ion trap. The linear ion trap is a series of quadrupole like rods with end cap electrodes. The same type of frequency variations used for the quadrupole are used with the charged electrodes to create a electromagnetic field which will trap the ions before they are moved to the detector.²⁷ This enables the selection and isolation of ions and ion fragments for further analysis and fragmentation.

1.3.7 Literature Search

The isolation and determination of secondary metabolites from natural sources is what natural products chemistry is all about. Using some or all of the separation and instrumentation methods discussed is required to isolate a pure compound and then correctly identify it. Because of the large amount of work that has already been done in the field of natural products chemistry there are many journals and databases containing information on compounds that have already been isolated. After gathering data such as substructures of your sample molecule and molecular formula a literature search of these journals and databases is the next step. The Dictionary of Natural Products is a great place to begin your search. It has an online database that is searchable by many fields and types of data as well as a substructure search feature. A literature search should always be performed in order to determine if the sample compound has already been isolated. Even if the sample compound has already been found it

may not have been identified from the sample species or genus or even family. There is a lot of diversity in secondary metabolites and even if the compound has been previously isolated from many sources it is still valuable information. Another source, especially if it is from another genus, family, or geographical region, is a helpful and welcome addition to the collective knowledge of the natural products community. Although there is a large number of isolated and identified secondary metabolites there is still so much more to discover and so many more natural sources that have yet to be analyzed. The forests and fields are full of life, the soil beneath our feet holds thousands of different strains of bacteria, and the oceans are a vast wilderness full of unanalyzed marine organisms. Although a lot of work has been done in the field of natural products, there is still so much left to discover if only the time and effort is put into finding it.

2 EXPERIMENTAL METHODS

2.1 General Experimental Procedures

All open column silica gel chromatography was performed using Silicycle SiliaFlash SiO₂ packing material with a particle size of 40-63 µm. All open column C₁₈ chromatography was performed with Silicycle Siliabond C₁₈ packing material with a particle size of 40-63 µm.

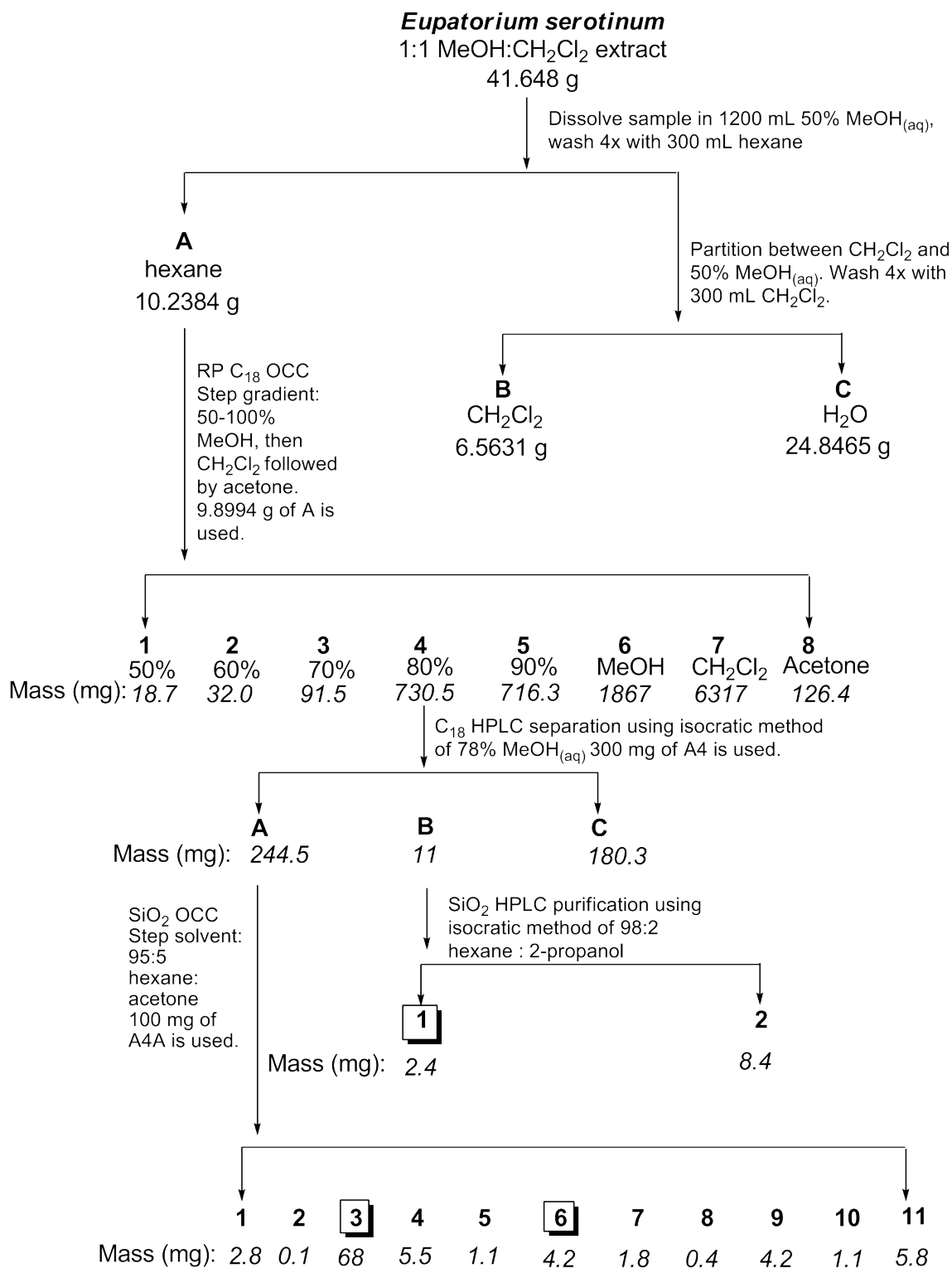
The C₁₈ HPLC was performed using a Varian Dynamax 250 mm by 21.4 mm Microsorb C₁₈ column. The pore size was 100 Å and the particle size was 5 µm. This column was used in conjunction with a Perkin-Elmer series 410LC pump and a Perkin-Elmer series 200 autosampler. The detector was a Perkin-Elmer LC-95 UV-Vis spectrophotometer detector set at 230 nm.

The normal phase (SiO₂) HPLC was performed using a Varian Dynamax Microsorb SiO₂ 250 mm by 10 mm column. The pore size was 100 Å and the particle size was 5 µm. The column was used with a Perkin-Elmer series 410LC pump and a FIAtron Ch-30 Column Heater set at 40 °C. The detector used was a Perkin-Elmer LC-95 UV/Vis spectrophotometer detector set at 210 nm.

2.2 Plant Material and Sample Preparation

Whole plant samples of *Eupatorium serotinium* were collected by Jason Clement and Kathy Mathews on August 26th, 2009. Kathy Mathews identified the plant. A voucher specimen was deposited in the Western Carolina University

Herbarium. The leaves of *Eupatorium serotinum* were air dried and ground into a powder. A crude organic extract of *Eupatorium serotinum* was produced by soaking 254 g of the ground plant material in 1.1 L of a 50:50 methanol : dichloromethane solvent system. The plant material was soaked overnight in the solvent system and then the solvent was dried from the extract by rotary evaporation. The extract was then partitioned using a modified Kupchan method. A total of 41.6 g of *Eupatorium serotinum* extract was dissolved in 1200 mL of the 50% methanol (aq) solvent system and was extracted with 4 washes of 300 mL of hexane. The hexane washes are pooled and dried (fraction A). The 50% methanol (aq) system was then extracted with 4 washes of 300 mL of dichloromethane. An emulsion formed during the extraction by dichloromethane and was allowed to dissipate before the next wash. The dichloromethane washes were pooled and dried (fraction B). The 50 methanol (aq) was concentrated under vacuum, lyophilized, and dried for storage (fraction C).

Figure 2.1: Fractionation tree for *Eupatorium serotinum*

2.3 Isolation of Secondary Metabolites

The combined hexane fraction, fraction A, was fractionated by a stepwise gradient using C₁₈ open column chromatography. A total of 244 g of C₁₈ was used in this column. The sample was dissolved in a small amount of hexane and loaded evenly onto the head of the C₁₈ column. The sample was then eluted off of the column with a stepwise gradient from 50% to 100% methanol (aq) in 10% increments, with 800 mL per wash followed by a dichloromethane wash and an acetone flush. These fractions were collected and dried. NMR analysis was performed on each fraction collected from this separation.

The material collected in the 80% methanol wash of the hexane partition, fraction A4, was selected as a candidate for C₁₈ HPLC. The reason for this was the preliminary ¹H NMR data collected for this fraction. The NMR data showed the presence of isopropyl groups as well as methyl signals and numerous signals in the alkene region. These signals are indicative of the presence of terpenoids, a family of chemical compounds that are common secondary metabolites. The next isolation step for fraction A4 used the preparatory HPLC column and an isocratic solvent system of 78% methanol (aq) with a flow rate of 10 mL per minute. The HPLC eluent was monitored using a UV-Vis detector set at 230 nm. A total of 435.8 mg out of the 730.5 mg collected for fraction A4 was used in the C₁₈ HPLC isolation step. Fraction A4A eluted first (244.5 mg) after 25 minutes and fraction A4B eluted second (11 mg) after 38 minutes.

The major peak, fraction A4A, by C₁₈ HPLC of the 80% methanol (aq) wash of the hexane partition was analyzed by TLC. Fraction A4A appeared to be one spot on the TLC plate under the UV lamp. However, after development using vanillin, the TLC visualizing agent, the TLC plate had at least 3 spots on the plate for fraction A4A. Two out of these three did not show up under the UV lamp but these 3 spots all produced a blue color when vanillin was used. This sample was selected for a SiO₂ gel open column separation using a 95:5 hexane : acetone as the mobile phase. The column consisted of 12.25 grams of SiO₂ gel. The column bed volume was measured to be 31 mL and 4 mL fractions were collected. A 100 mg aliquot of the sample was carefully loaded onto the column and eluted off with 200 mL of the 95:5 hexane : acetone solvent system. The column was then flushed with 200 mL of a 95:5 methanol : dichloromethane solvent system followed by a 50:50 methanol : dichloromethane solvent system in order to collect any sample material that might be left on the column. The collected fractions were then recombined based on TLC analysis. This separation afforded two compounds, A4A3 (68 mg) and A4A6 (4.2 mg) in sufficient purity and quantity for complete identification.

Fraction A4B by C₁₈ HPLC of the 80% methanol wash of the hexane partition was further purified by SiO₂ HPLC. A total of 10.8 mg of sample was used in this isolation step. An isocratic method of 98:2 hexane : 2-propanol was used, and the major peak was collected. This separation yielded compound A4B1 (2.4 mg) in sufficient purity and quantity for complete identification.

3 IDENTIFICATION OF SECONDARY METABOLITES FROM *EUPATORIUM SEROTINUM*

3.1 Identification of Compound A4A3

After isolating A4A3 by open column silica chromatography, it was analyzed by ^{13}C and ^1H NMR, IR, and liquid chromatography-electrospray ionization mass spectroscopy (LC-ESI MS). The data gathered showed that the sample contained a pure compound with a structure of 15 carbon atoms, which was determined by the ^{13}C NMR data. The proton NMR data suggested 22 hydrogen atoms. The LC-ESI MS data gives a pseudomolecular ion $[\text{M}+\text{H}]^+$ at m/z 219, Suggesting a nominal mass of 218 for A4A3. The MS data coupled with the ^{13}C and proton NMR data indicated a molecular formula of $\text{C}_{15}\text{H}_{22}\text{O}$, this matches the nominal mass of 218. The unsaturation number was calculated to be 5 based on the molecular formula. Upon closer inspection of the ^{13}C and DEPT-135 NMR spectra the signal at 208 ppm indicates the presence of a ketone functional group, which is consistent with the molecular formula. The signals at 176.5 ppm and 140.4 ppm represent the possible presence of alkene functional groups. Since there are only two possible alkene signals, and they both show HMBC correlation to the proton signal at 1.70 ppm, these carbons are bonded together. The chemical shift for these carbons are higher than expected, especially the carbon at 176.5 ppm. The main factor for this is the seven membered ring in the molecular structure. The HMBC data shows that the ^{13}C signal at 208 ppm and the signal at 176.5 ppm both correlate to the hydrogen peaks at 2.49 ppm and 1.70 ppm. This means that the ketone and the double

bond had to be in close proximity and helped to form the proposed substructure (Figure 3.1). Using this data, an α,β -unsaturated ketone substructure was proposed for the compound. The IR data includes a peak at 1691 cm^{-1} which also indicates an α,β -unsaturated ketone substructure. Inspection of the proton NMR spectrum revealed a methyl singlet whose signal was not split by any other hydrogen atoms at 1.00 ppm. The fact that there was only carbon NMR evidence for 2 double bonds and an unsaturation number of 5 meant that the molecule should have 3 rings in its structure. The molecular formula and substructure information was entered into the Dictionary of Natural Products search engine and the results were inspected for matches that had 3 rings. A literature search was conducted on the compounds that contained three rings and eventually ^{13}C NMR data from the literature for one of the compounds corresponded well with the data for A4A3.

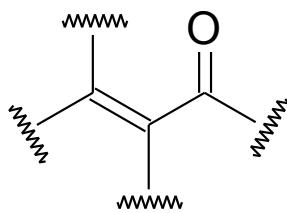


Figure 3.1: Substructure
for A4A3

Table 3.1: ^{13}C and ^1H NMR Data for A4A3

# C	δ_{C}	Literature δ_{C}	DEPT- 135	δ_{H}	Literature δ_{H}	Multiplicity
1	42.6	42.8	CH	2.95	3.06	m
2	40.3	40.5	CH ₂	2.49	2.39	dd
				2.05	1.95	dd
3	208.4	206.6	C	-	-	-
4	140.4	140.6	C	-	-	-
5	176.6	175.2	C	-	-	-
6	28.6	28.9	CH	1.45	1.54	m
7	32.4	32.8	CH	1.28	1.29	m
8	21.2	21.7	CH ₂	1.94	1.93	m
				1.61	1.68	m
9	32.6	33.3	CH ₂	2.08	2.09	m
				1.44	1.43	m
10	31.8	32.4	CH	2.02	2.02	m
11	26.1	26.1	C	-	-	-
12	16.6	16.7	CH ₃	1.00	1.05	s
13	29.6	29.2	CH ₃	1.25	1.25	s
14	17.6	17.7	CH ₃	0.79	0.8	d
15	8.3	8.2	CH ₃	1.70	1.66	d

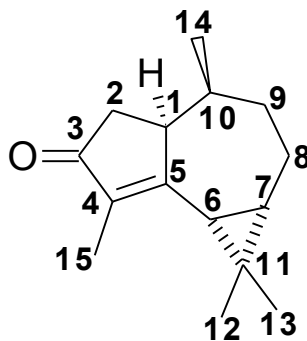


Figure 3.2: 4-Aromadendren-3-one

The compound was determined to be 4-aromadendren-3-one.²⁸ This compound has been isolated from several sources. Most of these are species of liverworts such as *Jubula japonica*,²⁹ *Symphyogyna brasiliensis*,³⁰ *Bazzania tridens*,³¹ and *Porella canariensis*.³² This compound has also been isolated from the Indonesian soft coral *Nephthea chabrolii*.³³ A comparison of the ¹³C NMR data from the article about the Indonesian soft coral with the carbon NMR data gathered on the sample shows very close correlation except for the ¹³C peak at 208 ppm, which shows up at 206 ppm in the literature reference.

Confirmation of the isolated compound's identity was accomplished by HMBC. The HMBC data shows correlation between the ¹³C 208.4 ppm signal of carbon C3 and the proton signals at 2.49 ppm, 2.05 ppm and 1.71 ppm. The signal at 1.71 ppm represents the methyl functional group of carbon C15. The proton signals at 2.49 ppm and 1.95 ppm represent the hydrogen atoms on carbon C2, which is adjacent to the ketone carbon C3. Another HMBC correlation that helps prove the identity of the compound is the correlation of the ¹³C signal at 26.1 ppm to the proton signals at 1.00 ppm, 1.26 ppm, 1.28 ppm, and 1.45 ppm. The ¹³C signal at 26.1 ppm is the quaternary carbon C11, which is in the three carbon ring. The proton signals at 1.00 ppm and 1.26 ppm belong to the methyl groups that are attached to the quaternary carbon. The proton signal at 1.45 ppm is for the hydrogen atom on carbon C6. The 1.28 ppm signal arises from the hydrogen on carbon C7. Another correlation in the HMBC spectra is the one that exists between the hydrogen atom connected to carbon C1, where the five and seven carbon rings are joined. This hydrogen atom produces a proton

signal at 2.95 ppm. This signal correlates to two ^{13}C signals, one at 17.6 ppm, which is for the methyl group on the seven carbon ring C14, and the other at 176.6 ppm, which is the quaternary carbon C5, where the five and seven carbon rings are joined.

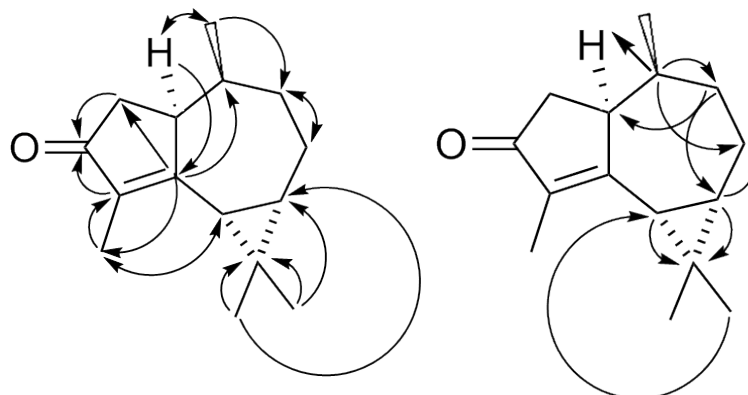


Figure 3.3: HMBC correlations for A4A3

Further confirmation of the identity of the compound can be attained from data gathered by COSY NMR. There is a correlation between the hydrogens on carbon C6 and carbon C7. There also exist correlations between the hydrogens on carbons C2 and C1, C1 and C10, C10 and C14, C9 and C10, and C8 and C9.

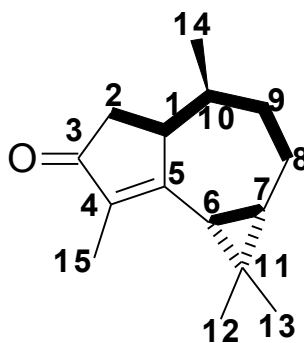


Figure 3.4: COSY correlations for A4A3

3.2 Identification of Compound A4A6

The second compound to be isolated from *Eupatorium serotinum*, A4A6, was isolated using the same open silica gel chromatography column as compound A4A3. The compound was analyzed by NMR, LC-ESI MS, and IR. The ^{13}C and DEPT-135 NMR data shows that there are 16 carbon signals. Two of these are quaternary carbons, as they only show up in the carbon spectrum and not the DEPT-135. Five of the carbon signals are indicative of methylene carbons. The proton NMR data suggests the presence of a meth-oxy group with a signal at 3.34 ppm. Using the HMBC data it was determined that both of the methyl groups at 0.94 ppm and 0.88 ppm correlate to the ^{13}C signal at 91.6 ppm. The HMBC data also shows that the methyl groups at 0.94 ppm and 0.88 ppm correlate to the ^{13}C signal at 31.5 ppm. The methyl group proton signal at 0.94 ppm corresponds to the ^{13}C signal at 21.12 ppm, which is for the carbon atom in the other methyl group attached to the carbon with the signal at 31.5 ppm. The signal at 0.88 ppm corresponds to the ^{13}C signal at 16.45 ppm, which is for the methyl group opposite it. These correlations indicate the substructure shown in Figure 3.5.

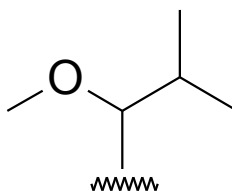


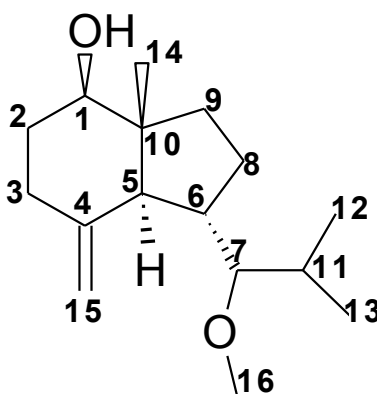
Figure 3.5: Substructure for A4A6.

The proton NMR data also suggests 28 protons in the molecular formula. The IR data gathered for the compound indicates that the compound contains an

alcohol functional group. The LC-ESI MS data shows a pseudomolecular ion $[M+H]^+$ peak at m/z 253, which would correspond to a nominal mass of 252. Using the NMR data and the LC-ESI MS spectra the molecular mass was determined to be 252, and the molecular formula to be $C_{16}H_{28}O_2$. Using the substructure in Figure 3.5, the molecular mass, and the molecular formula to search the Dictionary of Natural Products brought up only one result. The ^{13}C NMR data gathered for the sample corresponded well with the data found in the literature for the structure shown.³⁴ The compound found is 7 δ -Methoxy-4(14)-oppositen-1 β -ol.

Table 3.2: ^{13}C and ^1H NMR Data for A4A6

# C	δ_{C}	Literature δ_{C}	DEPT- 135	δ_{H}	Literature δ_{H}	Multiplicity
1	79.5	79.5	CH	3.58	3.57	d
2	32.0	35.1	CH ₂	1.89	1.90	m
				1.48	1.50	m
3	35.1	32.1	CH ₂	2.06	2.05	m
				2.38	2.40	m
4	146.2	146.2	C	-	-	-
5	55.5	55.5	CH	1.85	1.84	s
6	39.1	39.2	CH	2.38	2.40	m
7	91.7	91.7	CH	2.78	2.79	d
8	26.3	26.3	CH ₂	1.89	1.90	m
				1.36	1.34	m
9	37.2	37.3	CH ₂	1.73	1.80	m
				1.39	1.39	m
10	49.5	49.6	C	-	-	-
11	31.5	31.5	CH	1.74	1.73	m
12	16.5	16.5	CH ₃	0.88	0.88	d
13	21.1	21.2	CH ₃	0.94	0.95	d
14	12.5	12.5	CH ₃	0.65	0.64	s
15	107.6	107.6	CH ₂	4.78	4.80	s
				4.86	4.84	s
16	60.9	60.9	CH ₃	3.34	3.37	s

Figure 3.6: Structure of 7 δ -Methoxy-4(14)-oppositen-1 β -ol.

There are several HMBC correlations that help confirm this assignment. One of them is a correlation that connects the substructure to the five carbon ring. The ^{13}C signal at 39.1 ppm is for carbon C6, and it correlates to the proton on carbon C11, where the two methyl groups are attached. This shows up on the proton spectra at 1.74 ppm. Another HMBC correlation is for the methylene group attached to the six carbon ring. The exocyclic sp^2 methylene protons give signals at 4.78 ppm and 4.86 ppm. These correlate to the ^{13}C signal at 55.5 ppm that is for carbon C5. There are also HMBC correlations for the methyl group that is attached to carbon C10. The proton signal for the methyl group, at carbon C14, shows up at 0.65 ppm, and correlates to several ^{13}C signals. The ^{13}C signals are 37.2 ppm for carbon C9, 49.5 ppm for the quaternary carbon C10, 55.5 ppm for the tertiary carbon C5, and 79.5 ppm for carbon C1, which has the alcohol attached.

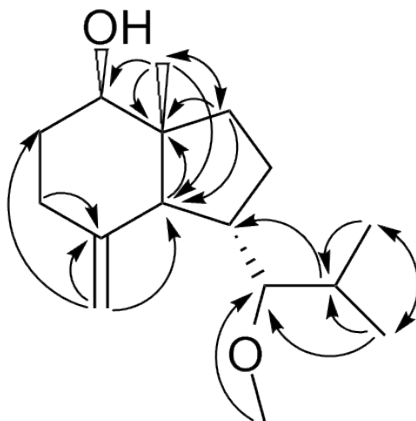


Figure 3.7: HMBC correlations for A4A6

There are also COSY NMR correlations that help confirm the structure. There is a correlation between the protons on carbons 1 and 2. There are also correlations that connect a large majority of the molecule. These correlations are between the protons on carbons 5 and 6, 6 and 7, 7 and 11, 11 and 12, and 11

and 13. These correlations help confirm the substructure as the ones regarding protons on carbon 6, 7, 11, 12, and 13 are all in the proposed substructure. The one between the protons on the carbons 4 and 15 help confirm the presence of the exocyclic sp^2 methylene.

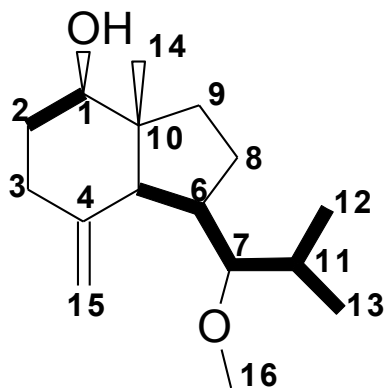


Figure 3.8: COSY correlations for A4A6

This compound has been found in *Saussurea pulchella*³⁵, *Aster scaber*³⁶, and *Torilis japonica* D.C.³⁷ Both *Saussurea pulchella* and *Aster scaber* are native to Korea and *Torilis japonica* is native to Japan. Both *Saussurea pulchella* and *Aster scaber* are from the Aster family (Asteraceae), which is the same family as *Eupatorium serotinum*.

3.3 Identification of Compound A4B1

The third compound isolated from *Eupatorium serotinium*, A4B1, was isolated by normal phase SiO₂ purification of the B fraction of the reverse phase C₁₈ HPLC separation. Determination of its structure was accomplished by the same methods as used for the other two compounds. The compound was analyzed by NMR, LC-ESI MS, and IR. The number of carbons in the molecular formula, 15, is determined by examining the ¹³C NMR spectra. The presence of an alcohol group, and therefore an oxygen, in the molecular formula is determined by the broad signal around 3300 in the IR data for the sample. The LC-ESI MS data shows a M+H peak at 221, giving a nominal mass of 220. Using this data the molecular formula is determined to be C₁₅H₂₄O. The unsaturation number for this compound was calculated to be 3. Examination of the DEPT-135 NMR spectra shows 2 quaternary carbons and 6 methylene carbons. The ¹³C spectra displays evidence of 4 double bonded carbons. Examination of the HSQC and HMBC spectra shows that one of the double bonded carbons is a methylene carbon that correlates with another double bonded carbon. The methylene carbon signal is at 113.2 ppm, and correlates to the proton signals at 4.9 ppm and 4.8 ppm on the HSQC spectra. The HMBC spectrum shows correlation between the 4.9 ppm and 4.8 ppm proton signals and the ¹³C signal at 129.9 ppm, this correlation connects the methylene functional group to an alkene. The other two double-bonded carbons are a methylene and a quaternary carbon as well. Based on this evidence it is determined that there are two geminal disubstituted double bonds in the molecule. One of them has signals at

146.8 ppm for the quaternary carbon and 113.2 ppm for the alkene carbon while the other is at 153.5 ppm for the quaternary carbon and 110.9 ppm for the alkene carbon. In addition to this the proton NMR spectra also shows evidence of an isopropyl group at 0.89 ppm and 0.81 ppm. These signals are for two methyl groups which couple to a methine to make the isopropyl group. Using the substructures (Figure 3.9) revealed in the NMR data, the alcohol group revealed by the IR data, and the molecular formula confirmed by the LC-ESI MS data, a search was carried out in the Dictionary of Natural Products. The resulting choices were narrowed down using the index of hydrogen deficiency calculated from the molecular formula of the compound. With an unsaturation number of 3 and 2 double bonds in the molecule there should only be one ring. After searching the literature and comparing ^{13}C NMR data a match was found.

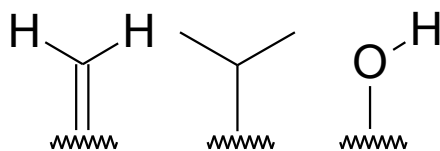
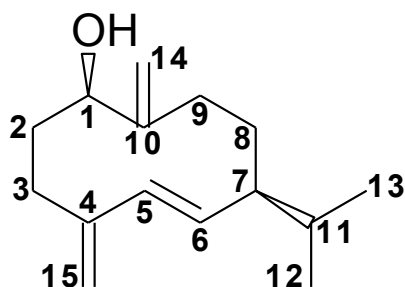


Figure 3.9: Substructures for A4B1

Table 3.3: ^{13}C and ^1H NMR Data for A4B1

# C	δ_{C}	Literature δ_{C}	DEPT-135	δ_{H}	Literature δ_{H}	multiplicity
1	76.9	76.0	CH	3.78	3.77	
2	36.5	36.2	CH ₂	1.67	1.7	m*
				2.06	2.04	m*
3	30.2	29.9	CH ₂	2.17	2.18	m
				2.44	2.43	dt
4	146.8	146.8	C	-	-	-
5	129.9	129.7	CH	6.02	6	d
6	138.3	138.0	CH	5.44	5.43	dd
7	52.8	52.5	CH	1.67	1.78	m*
8	36.6	36.1	CH ₂	2.05	2.04	m*
				1.63	1.63	m*
9	34.8	34.5	CH ₂	1.66	1.65	m*
				2.61	2.63	m
10	153.5	153.5	C	-	-	-
11	32.1	31.8	CH	1.49	1.49	m
12	21.1	20.7	CH ₃	0.91	0.89	d
13	20.8	20.5	CH ₃	0.80	0.81	d
14	110.9	110.6	CH ₂	5.27	5.27	s
				5.00	5	s
15	113.2	112.9	CH ₂	4.92	4.92	s
				4.85	4.84	s

* multiplicity is obscured by other signals

Figure 3.10: Germacra-4(15),5,10(14)-trien-1 β -ol

The compound was determined to be germacra-4(15),5,10(14)-trien-1 β -ol. This structure is supported by an HMBC spectra correlation between the ^{13}C signal at 52.8 ppm and the protons on the isopropyl group at 0.81 ppm and 0.89 ppm. There is also a correlation between the protons on the methylene group, C14, and carbons C1 and C9 to either side of the methylene group. The methylene proton signals are at 5.00 ppm and 5.27 ppm. The carbon signals they correlate to are at 76.9 ppm for C1, which the alcohol is attached to, and 34.8 ppm for C9, which is the other adjacent carbon.

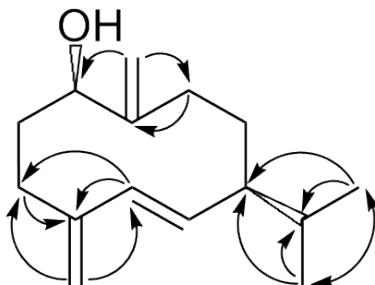


Figure 3.11: HMBC correlations for A4B1

The COSY NMR data also helps to confirm the identity of the sample. There are several COSY correlations observed for the sample. Two correlations exist between the protons on carbons C1 and C2 and carbons C2 and C3. There is a correlation between the protons on carbons C5 and C6 as well as on the protons on carbon C8 and C9. The last sets of COSY correlation confirm the presence of the isopropyl group, and are between the protons on carbons C11 and C12 and C11 and C13.

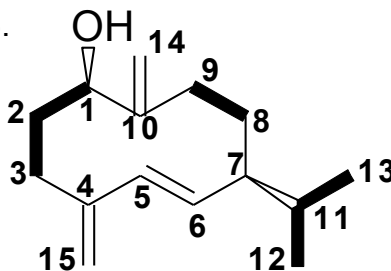


Figure 3.12: COSY correlations for A4B1

Germacra-4(15),5,10(14)-trien-1 β -ol was been found in a wide variety of plants including *Artemisia annua*,³⁸ *Artemisia stolonifera*,³⁹ *Bupleurum spinosum*,⁴⁰ *Inula cuspidate*,⁴¹ *Aster scaber*,⁴² *Garcinia scortechinii*,⁴³ *Eryngium yuccifolium*,⁴⁴ *Echinacea purpurea*,⁴⁵ *Laurus nobilis*,⁴⁶ *Heterothalamus alienus*,⁴⁷ *Senecio glanduloso-pilosus*,⁴⁸ *Solidago virga-aurea* var. *gigantea*,⁴⁹ *Mikania pohlii*,⁵⁰ and *Senecio confusus*.⁵¹ Members of the Aster family containing this compound include *Artemisia annua*, *Artemisia stolonifera*, *Inula cuspidate*, *Aster scaber*, *Echinacea purpurea*, *Heterothalamus alienus*, *Senecio glanduloso-pilosus*, *Solidago virga-aurea* var. *gigantea*, *Mikania pohlii*, and *Senecio confusus*. It has also been synthesized as an intermediate for periplanones C and D.⁵² It also showed some moderate anti-fungal activity against *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Epidermophyton floccosum*.⁵³

3.4 Conclusion

From an extract of *Eupatorium serotinum* three compounds were isolated by a modified Kupchan separation followed by several chromatographic separations including open column and HPLC methods on both C₁₈ and stand SiO₂ materials. These compounds were previously unknown in this plant, but have all been discovered in other species. These compounds are 4-aromadendren-3-one (A4A3), 7 δ -methoxy-4(14)-oppositen-1 β -ol (A4A6), and germacra-4(15),5,10(14)-trien-1 β -ol (A4B1). The three compounds discovered in *Eupatorium serotinum* raise the total of known compounds for this plant from five

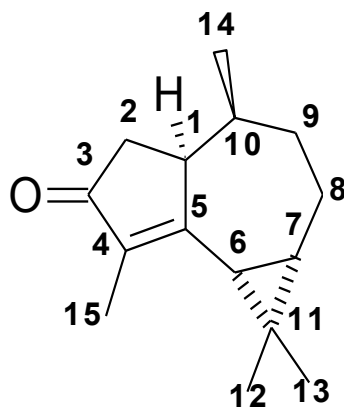
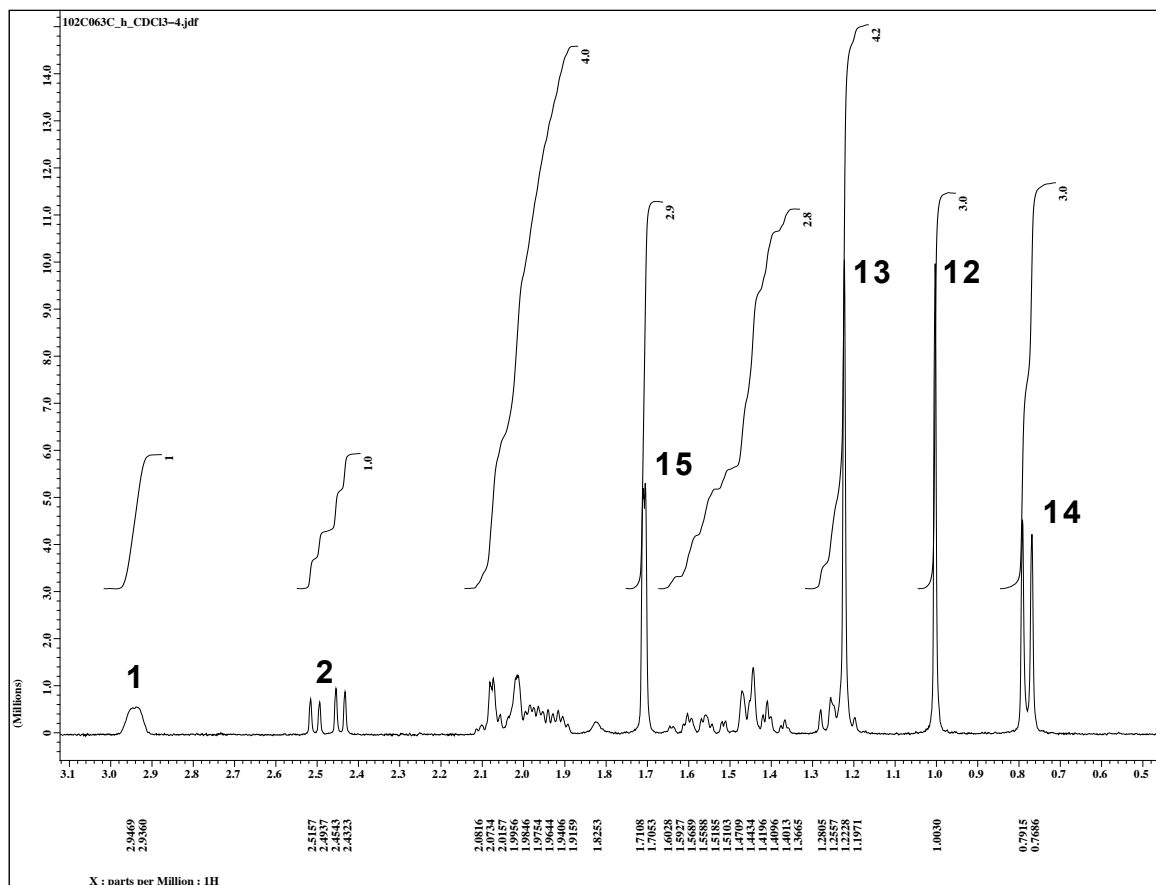
to eight. Both 7 δ -methoxy-4(14)-oppositen-1 β -ol, and germacre-4(15),5,10(14)-trien-1 β -ol have been previously discovered in species that come from the Aster family (Asteraceae), which is the same family as *Eupatorium serotinum*. These two compounds might serve in the future as biomarkers for the Aster family.

Biomarkers help scientists who are presented with a sample from an unknown source to determine from what or where it came. The other compound discovered, 4-aromadendren-3-one, has been discovered primarily in liverworts.

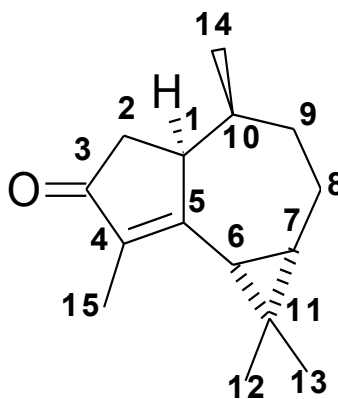
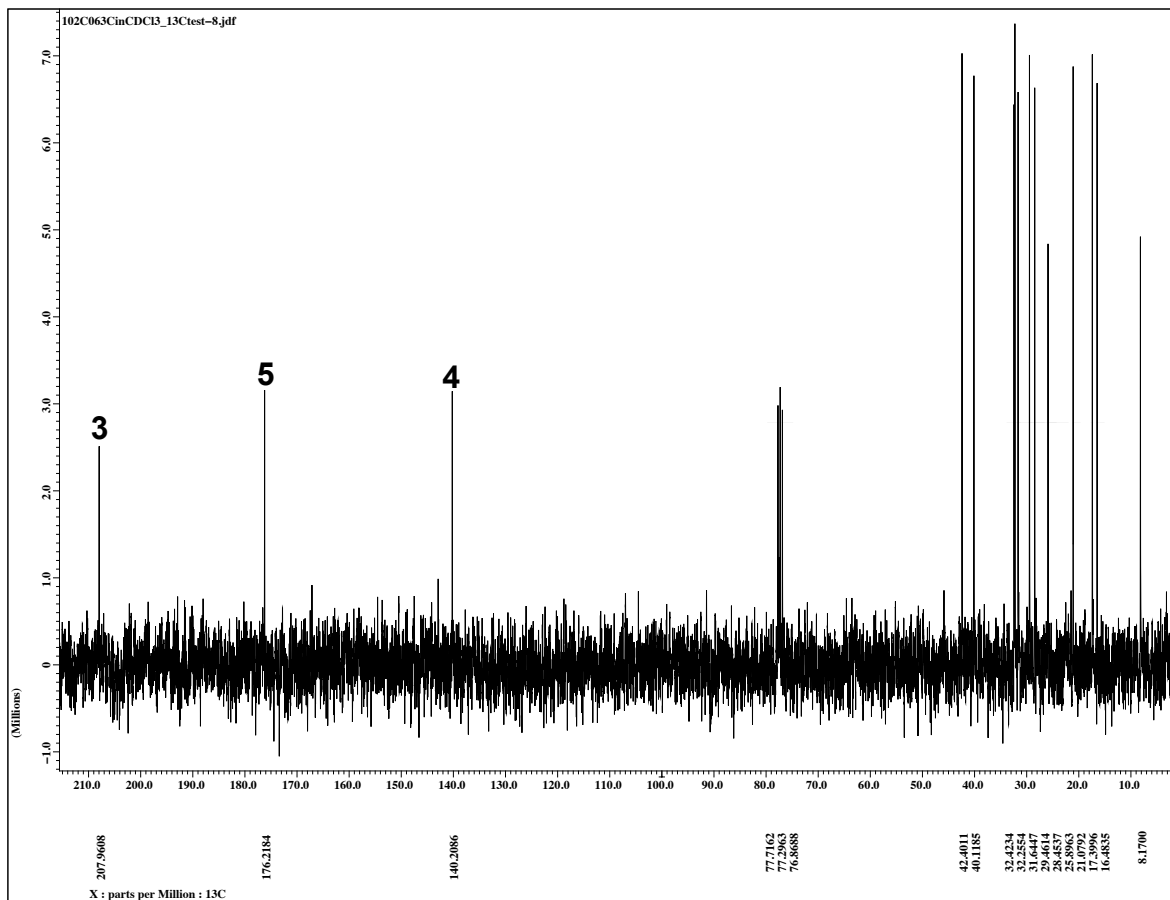
4 DATA COLLECTED FROM THE PURIFIED SAMPLES.

4.1 Data Collected from A4A3

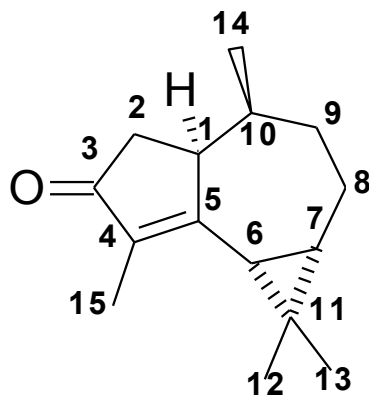
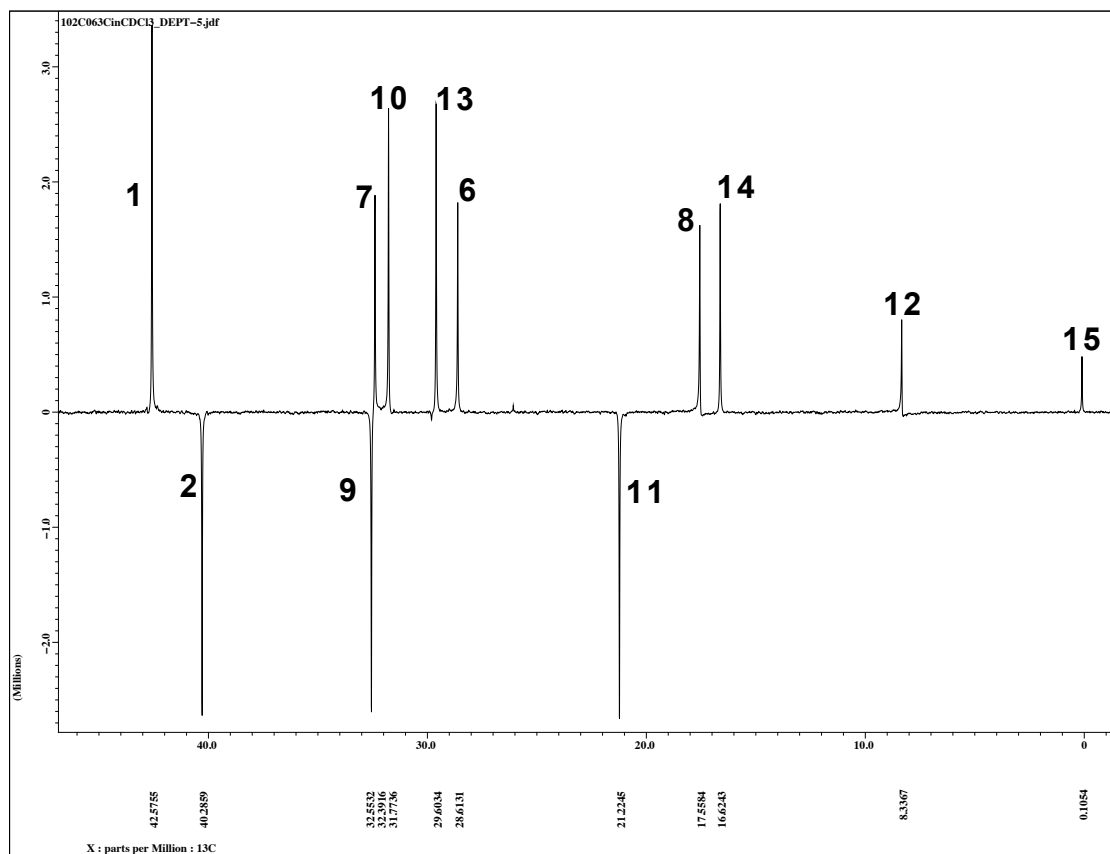
4.1.1 Proton NMR Data Collected from A4A3



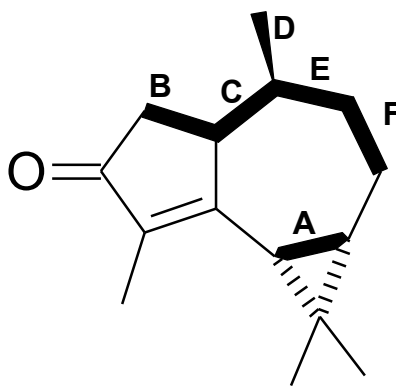
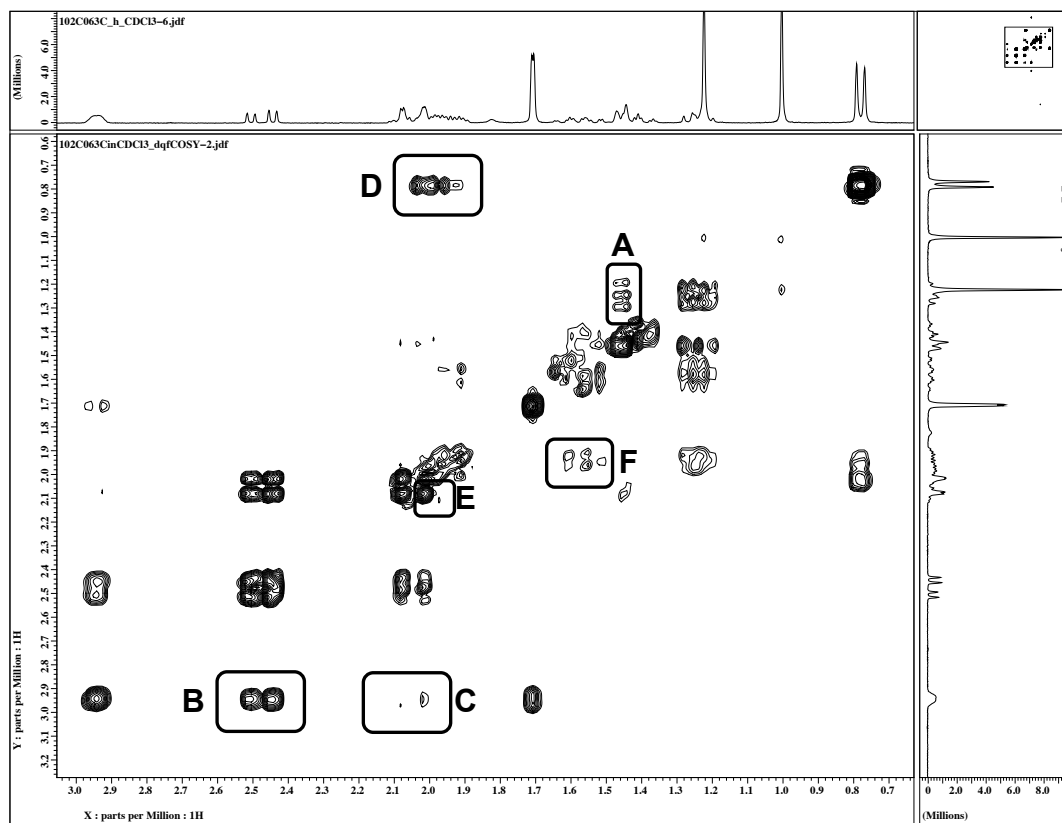
4.1.2 ^{13}C NMR Data Collected from A4A3



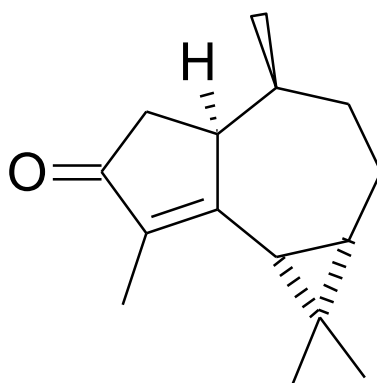
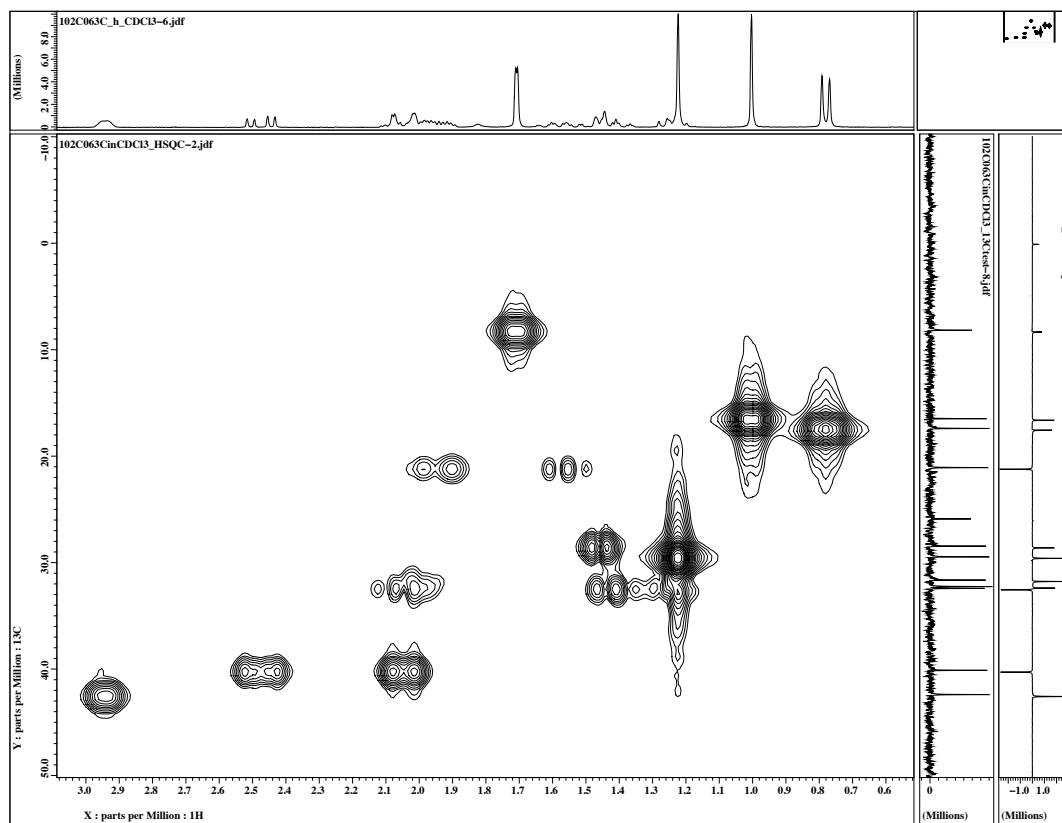
4.1.3 DEPT-135 NMR Data Collected from A4A3



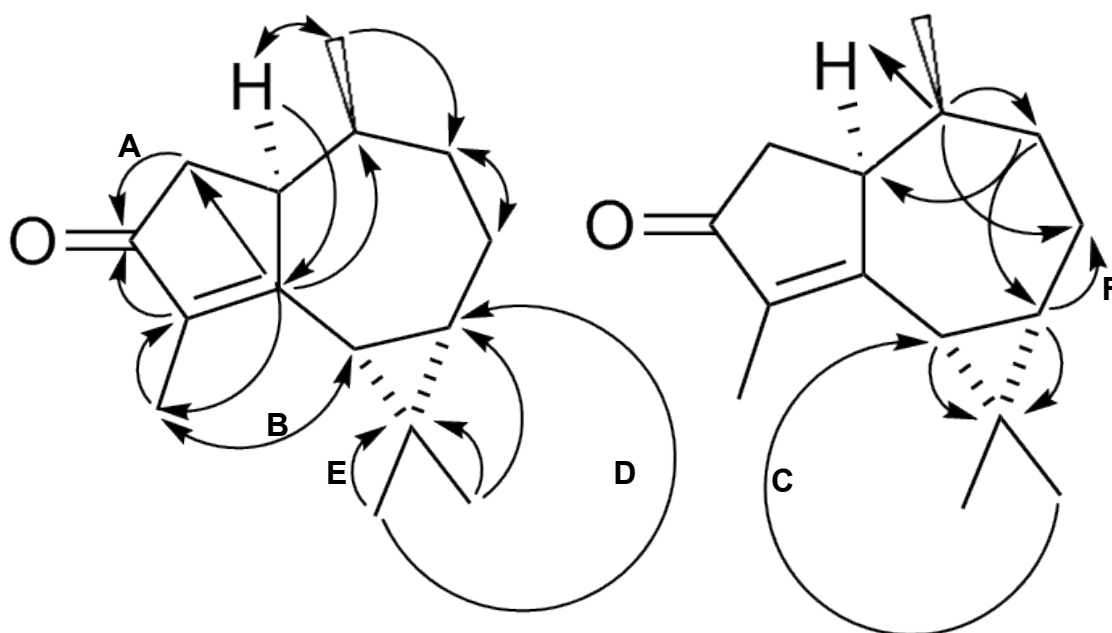
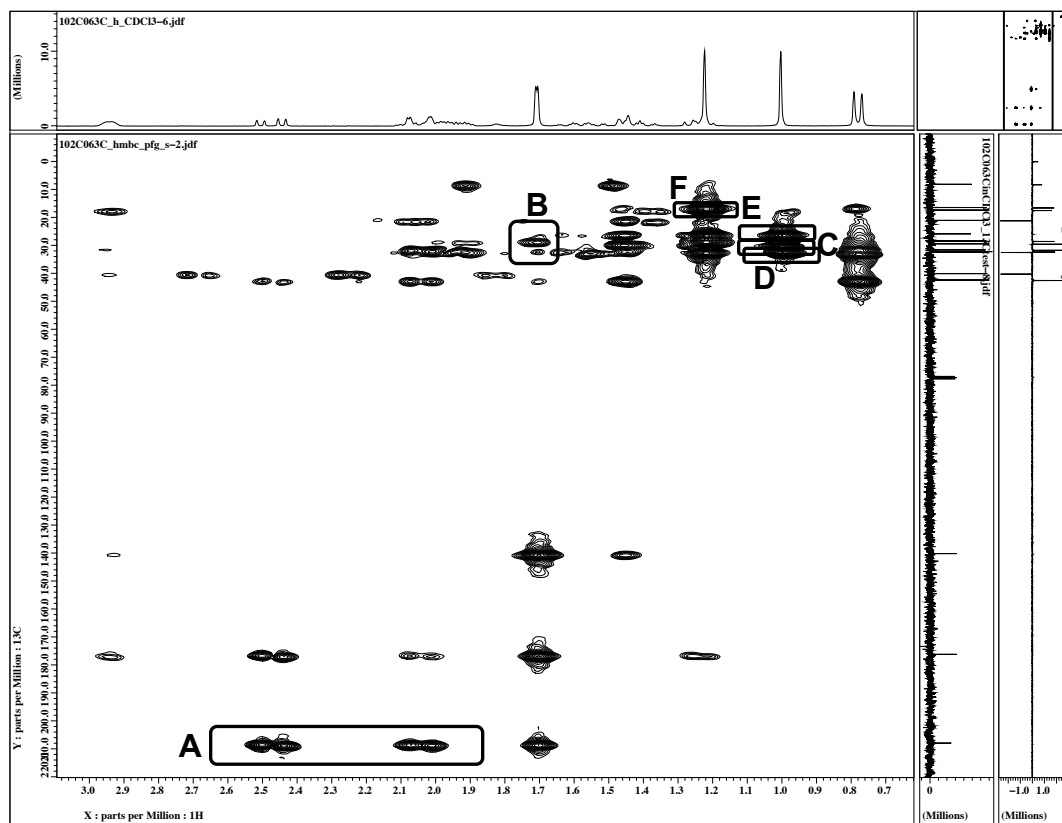
4.1.4 COSY NMR Data Collected from A4A3



4.1.5 HSQC NMR Data Collected from A4A3



4.1.6 HMBC NMR Data Collected from A4A3

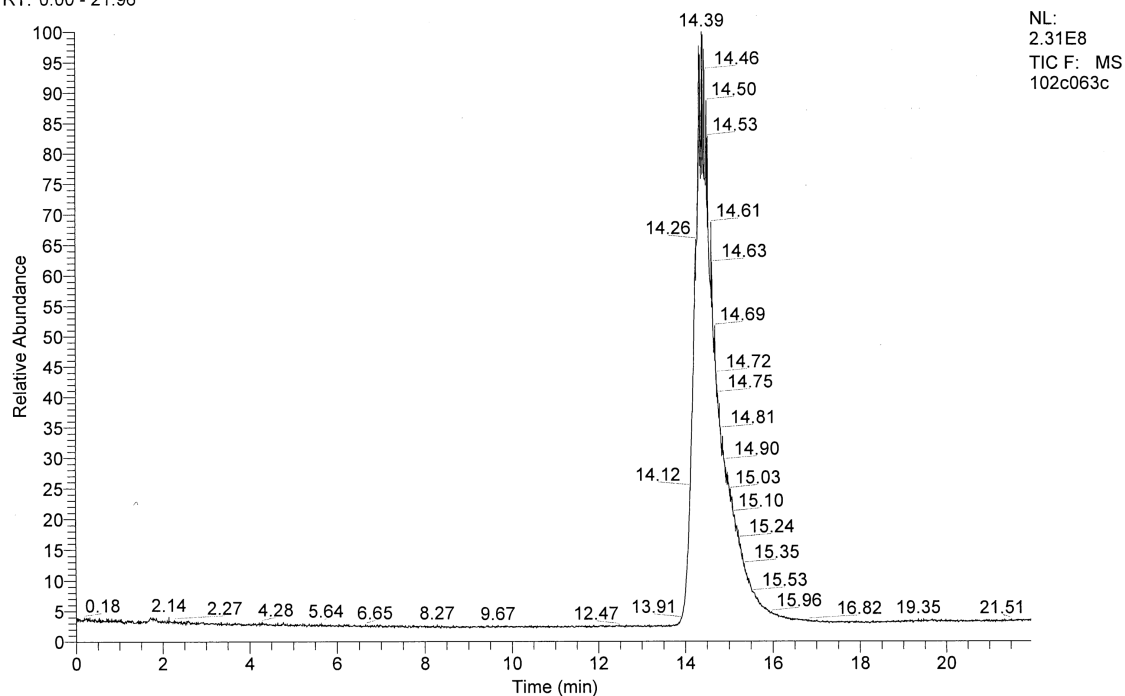
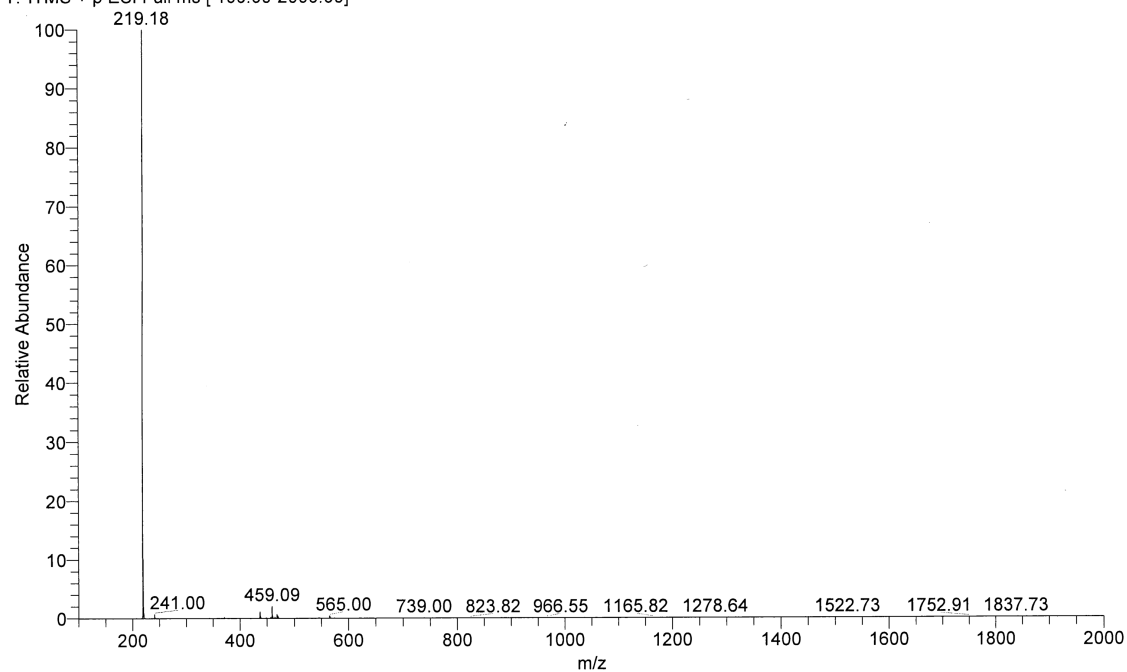


4.1.7 ESI-MS Data Collected from A4A3

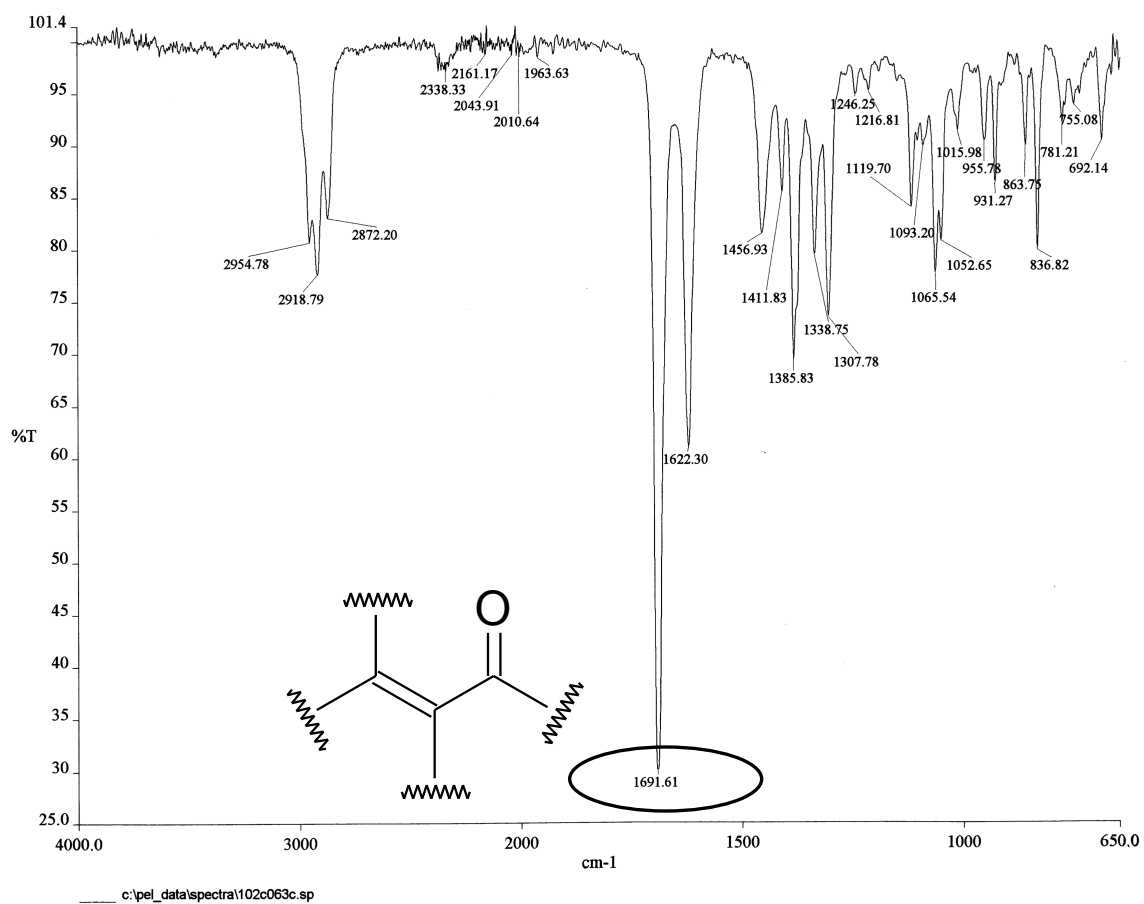
C:\Xcalibur\data\Clement\102c063c

7/22/2011 12:43:21 PM

RT: 0.00 - 21.96

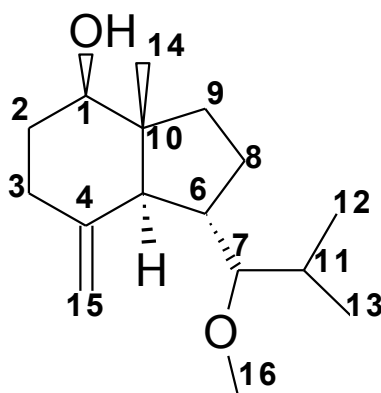
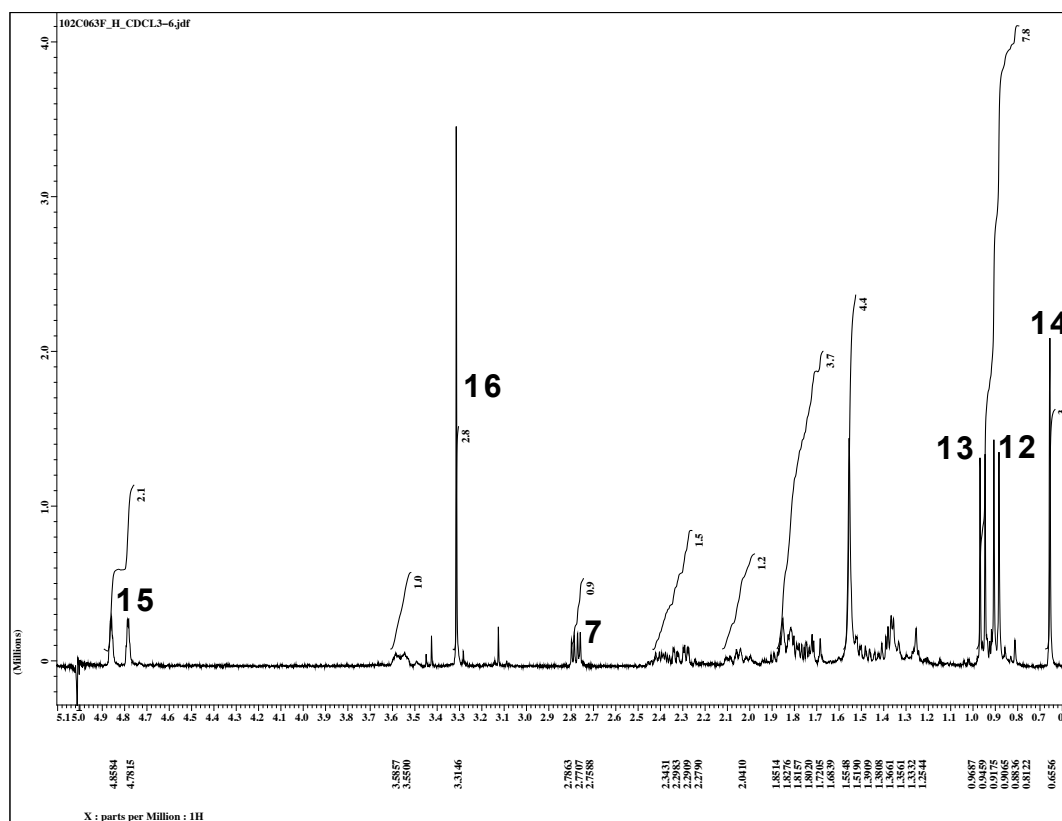
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T: ITMS + p ESI Full ms [100.00-2000.00]

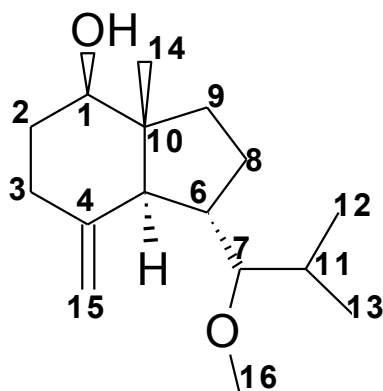
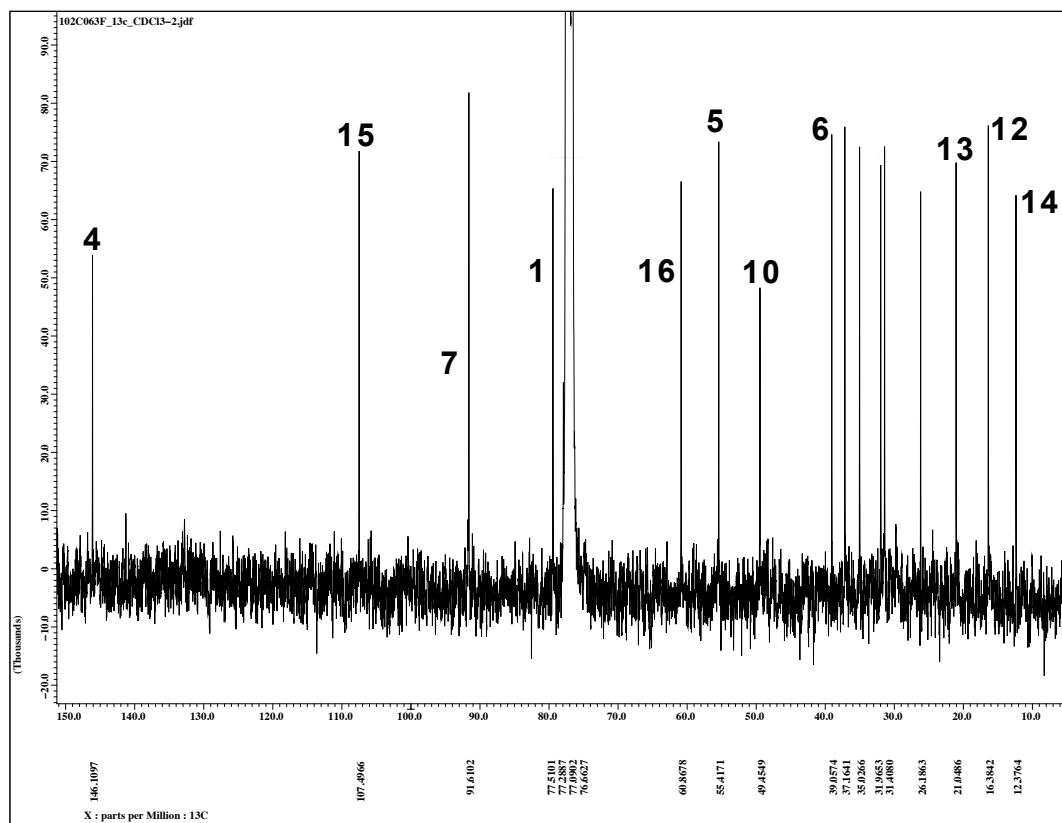
4.1.8 IR Data Collected from A4A3



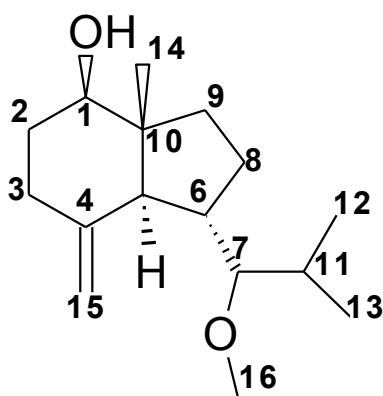
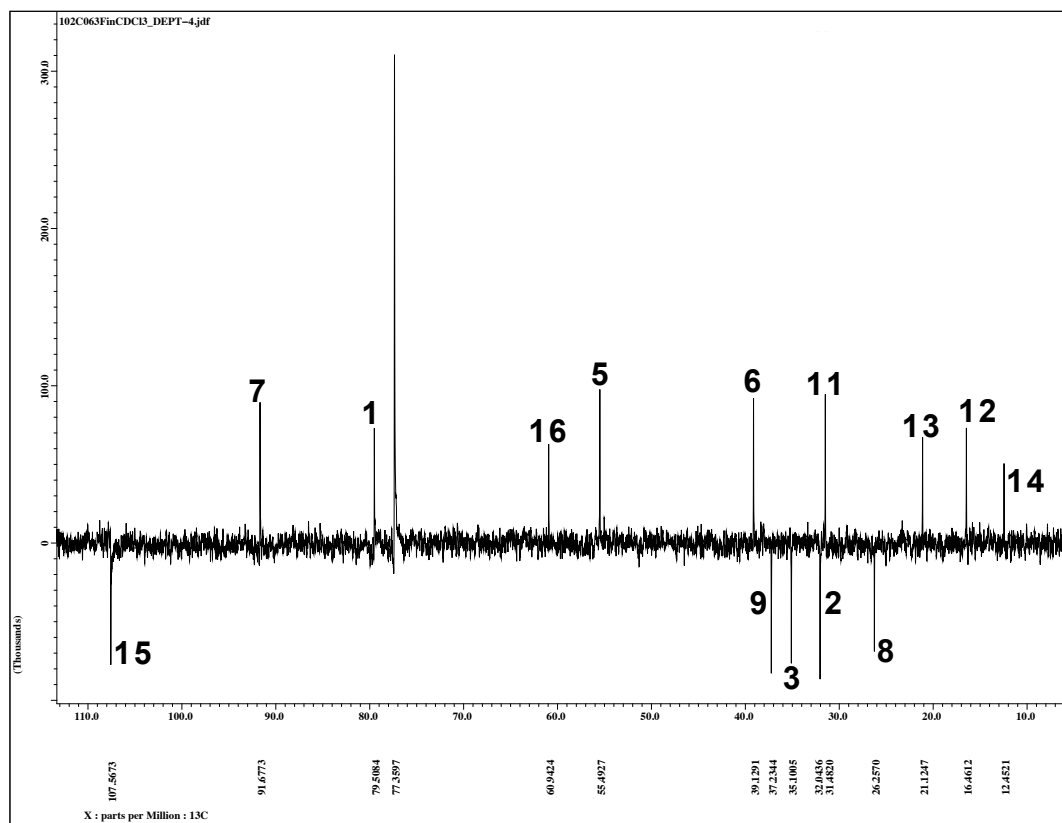
4.2 Data Collected from A4A6

4.2.1 Proton NMR Data Collected from A4A6

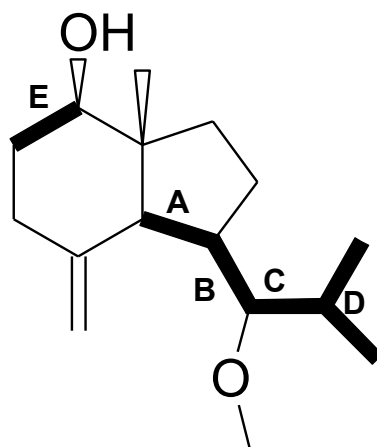
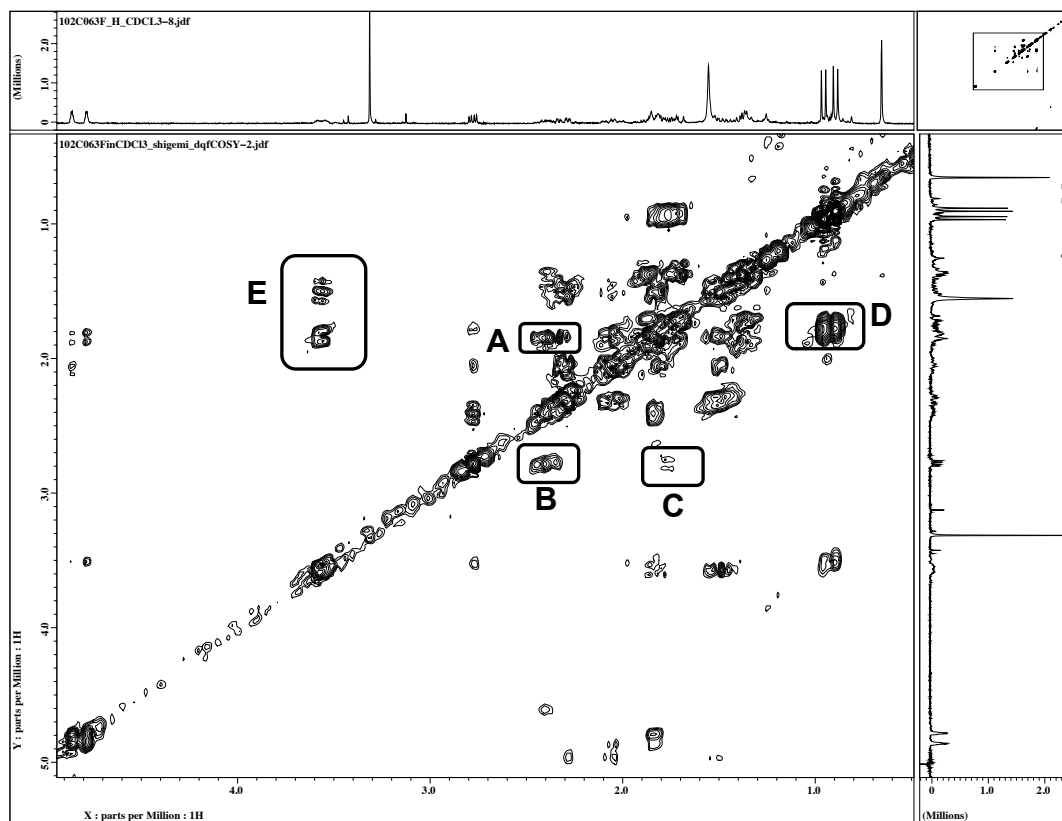


4.2.2 ^{13}C NMR Data Collected from A4A6

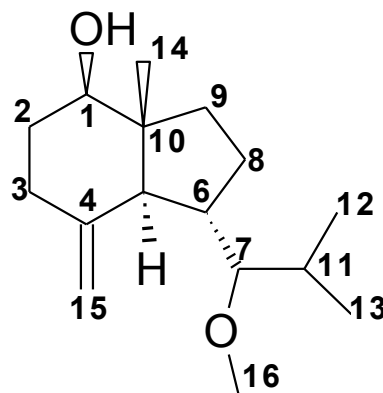
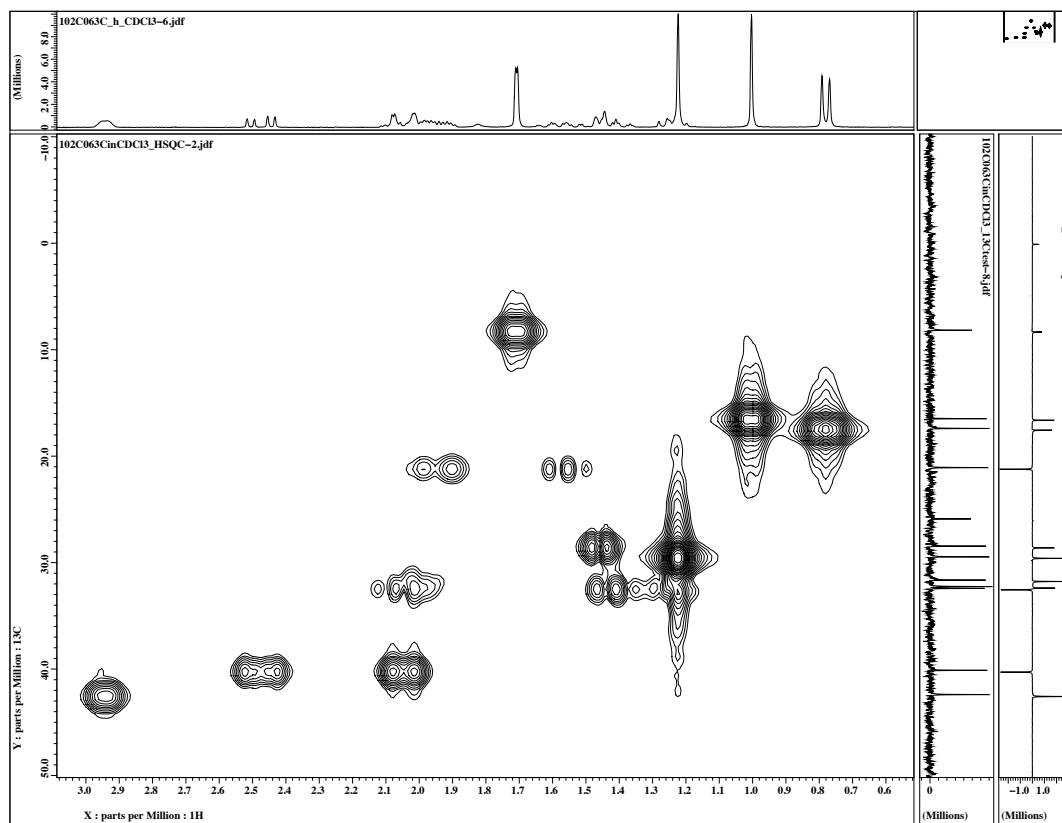
4.2.3 DEPT-135 NMR Data Collected from A4A6



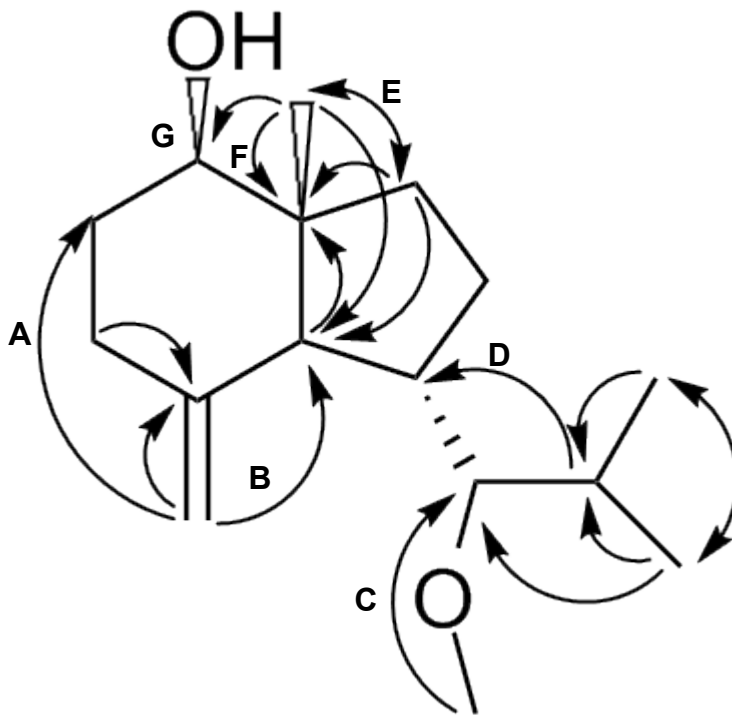
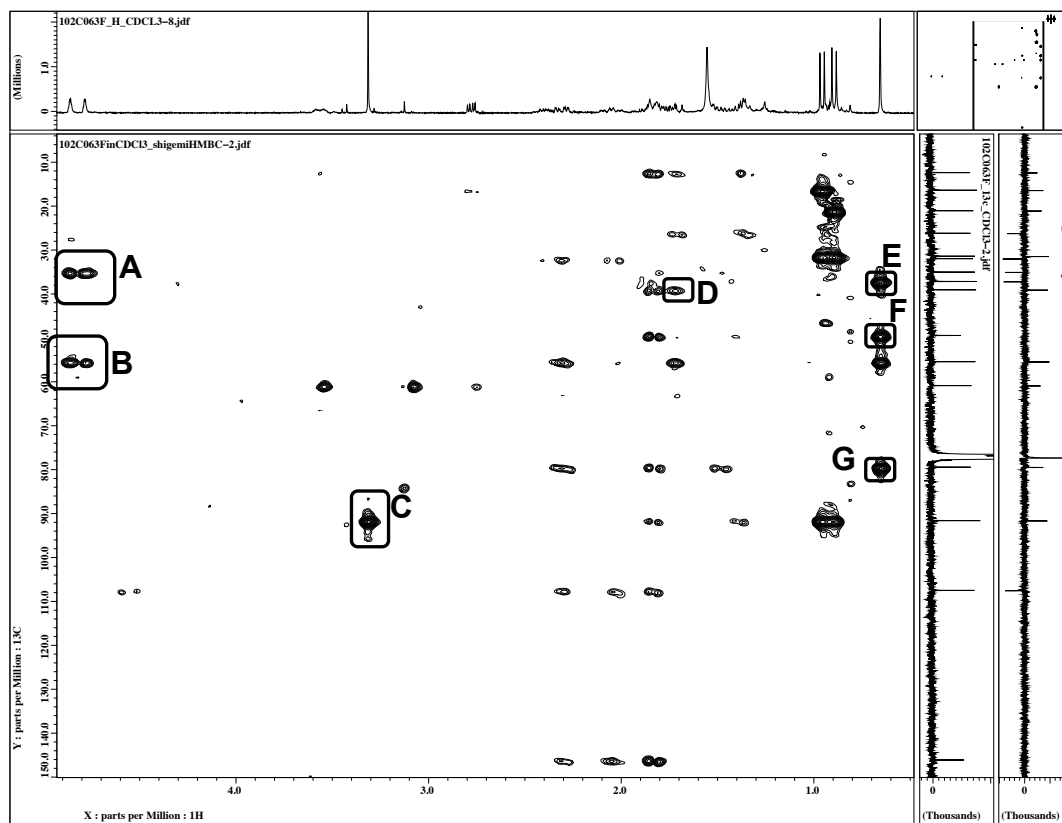
4.2.4 COSY NMR Data Collected from A4A6



4.2.5 HSQC NMR Data Collected from A4A6



4.2.6 HMBC NMR Data Collected from A4A6

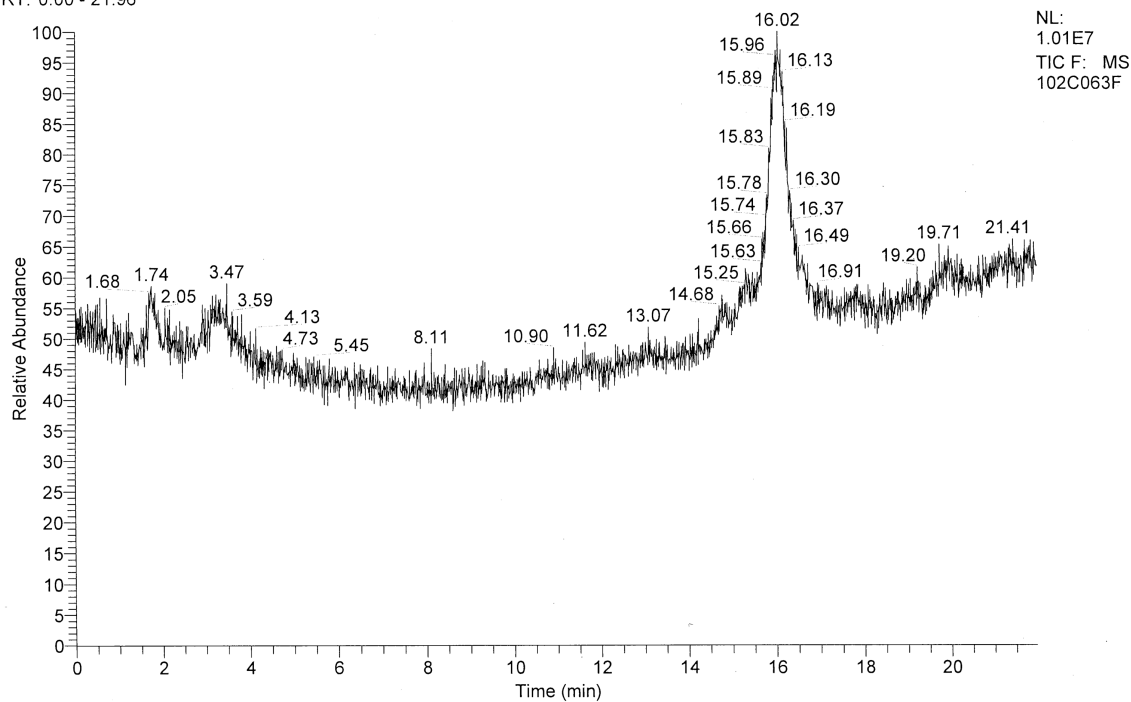
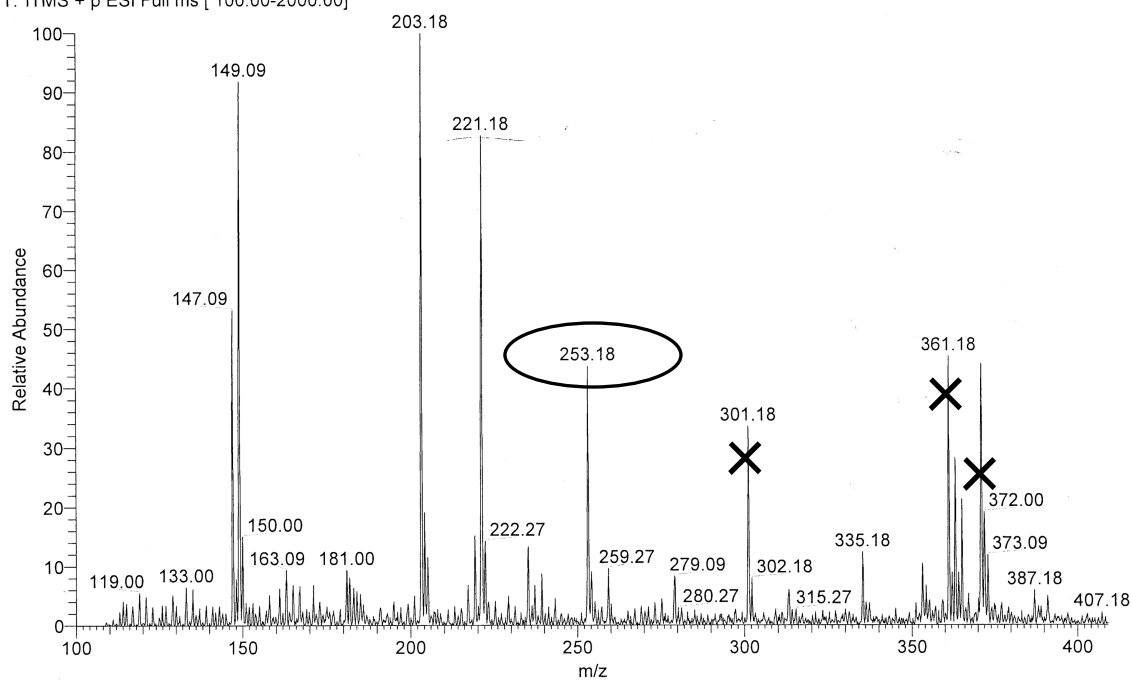


4.2.7 ESI-MS Data Collected from A4A6

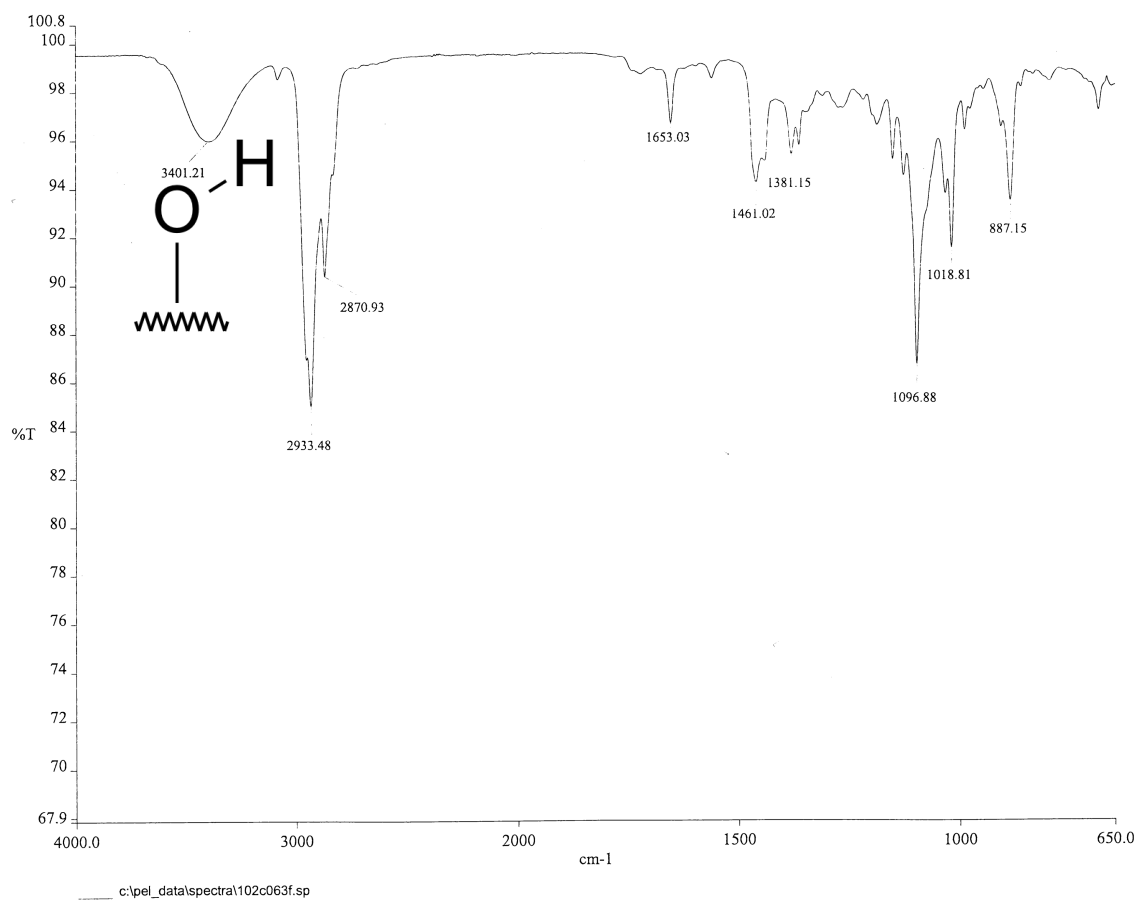
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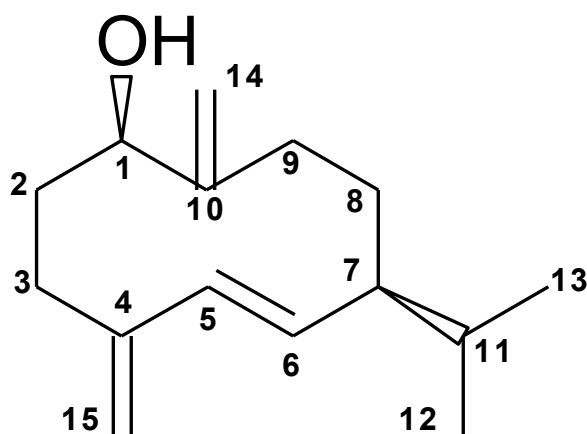
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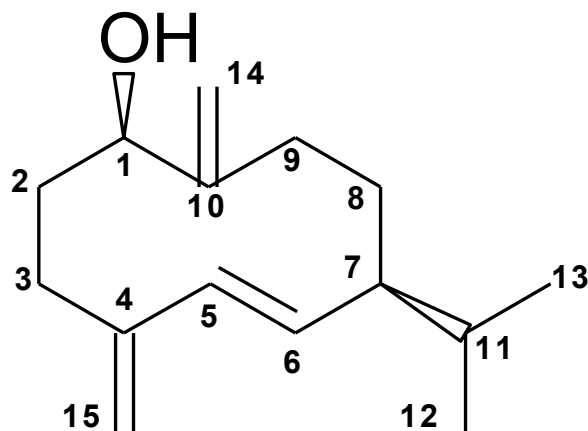
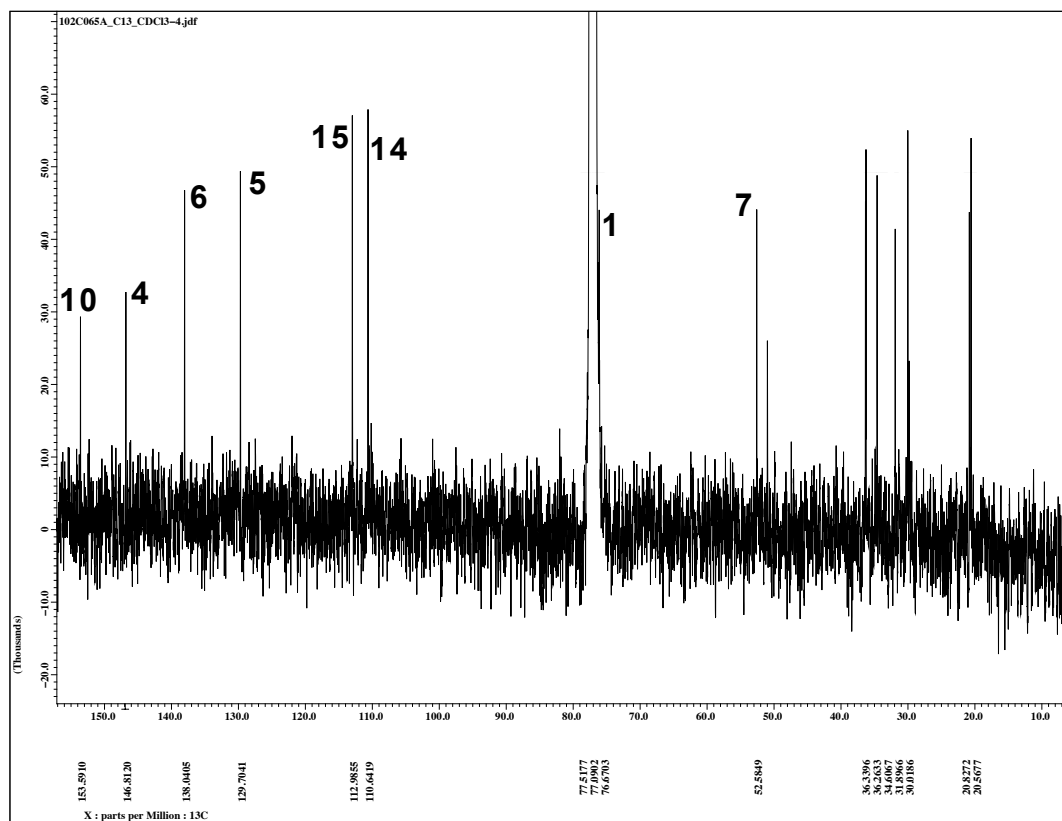
RT: 0.00 - 21.96

102C063F #2201 RT: 16.00 AV: 1 NL: 1.25E5
T: ITMS + p ESI Full ms [100.00-2000.00]

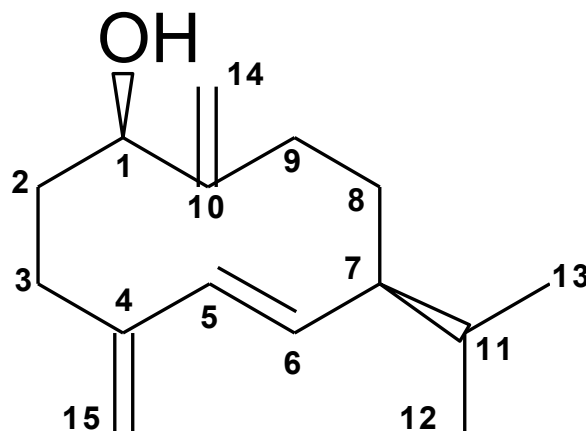
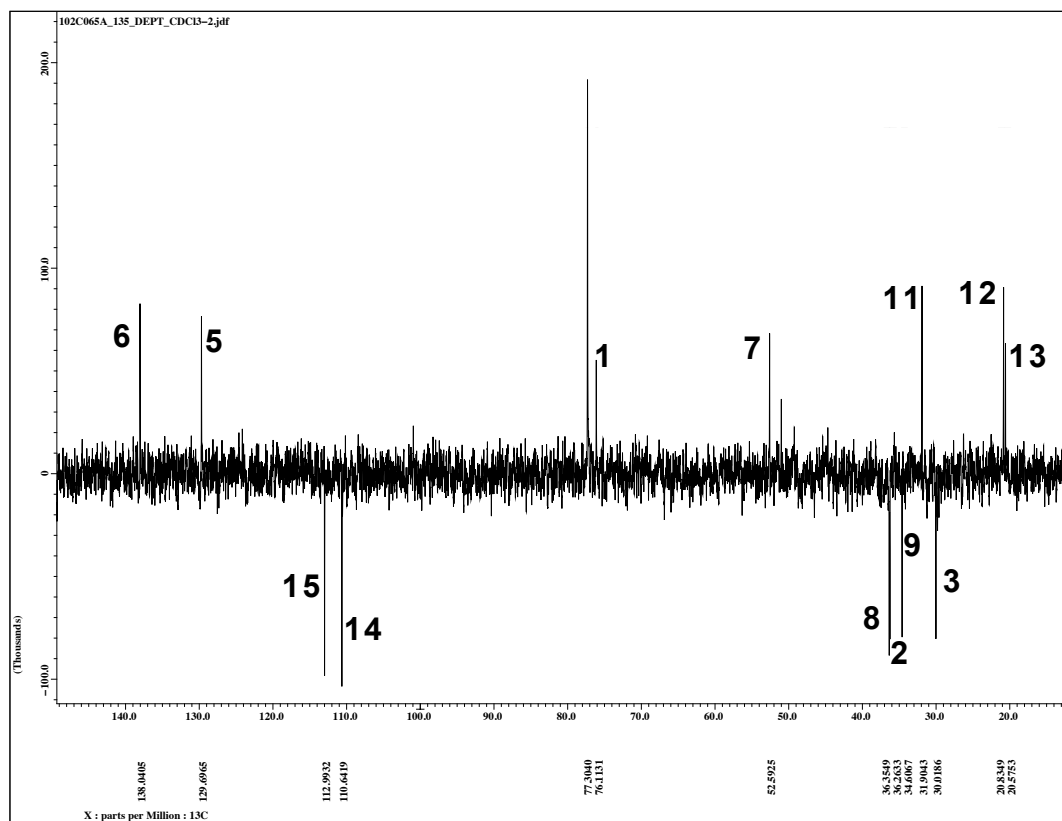
4.2.8 IR Data Collected from A4A6



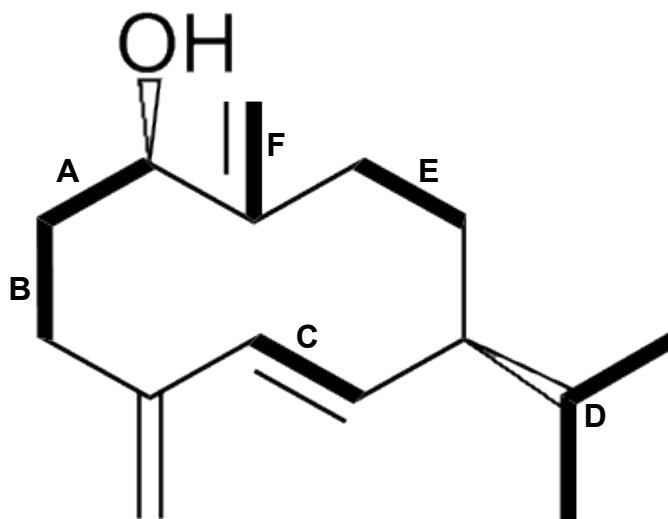
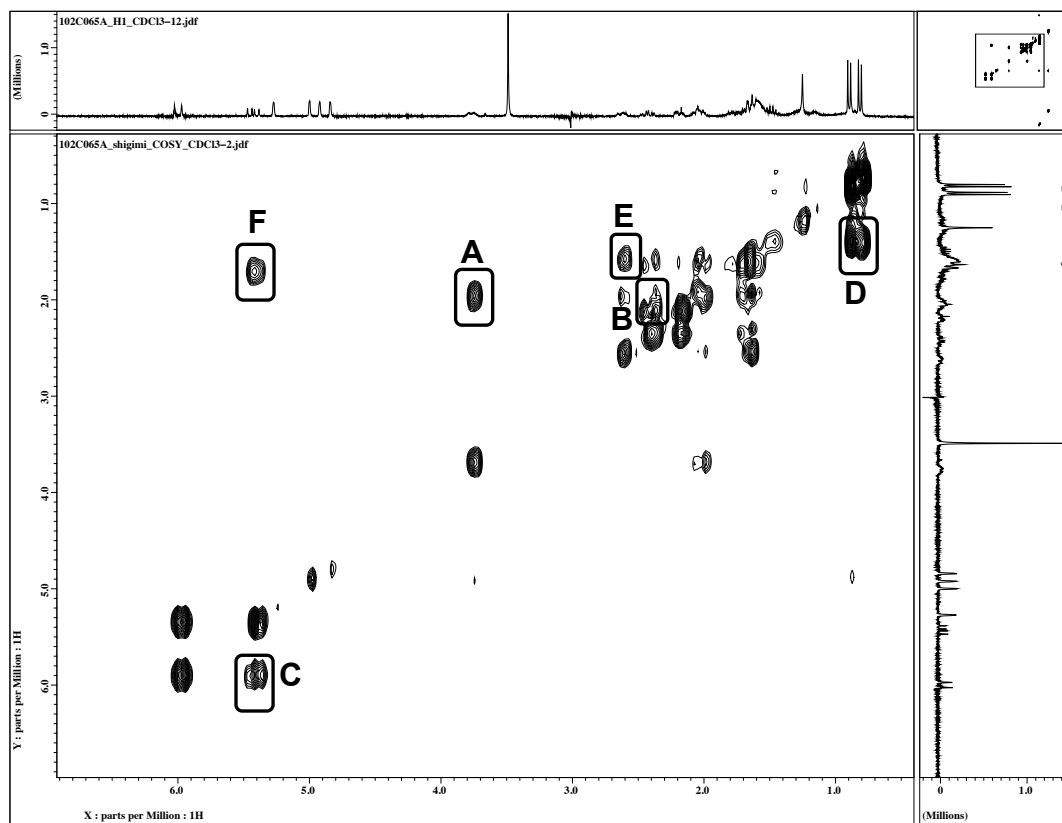


4.3.2 ^{13}C NMR Data Collected from A4B1

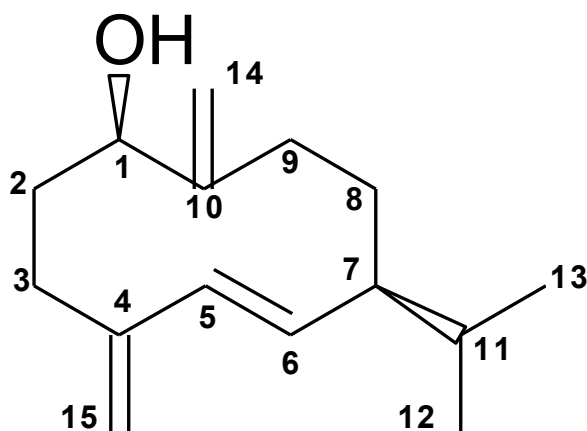
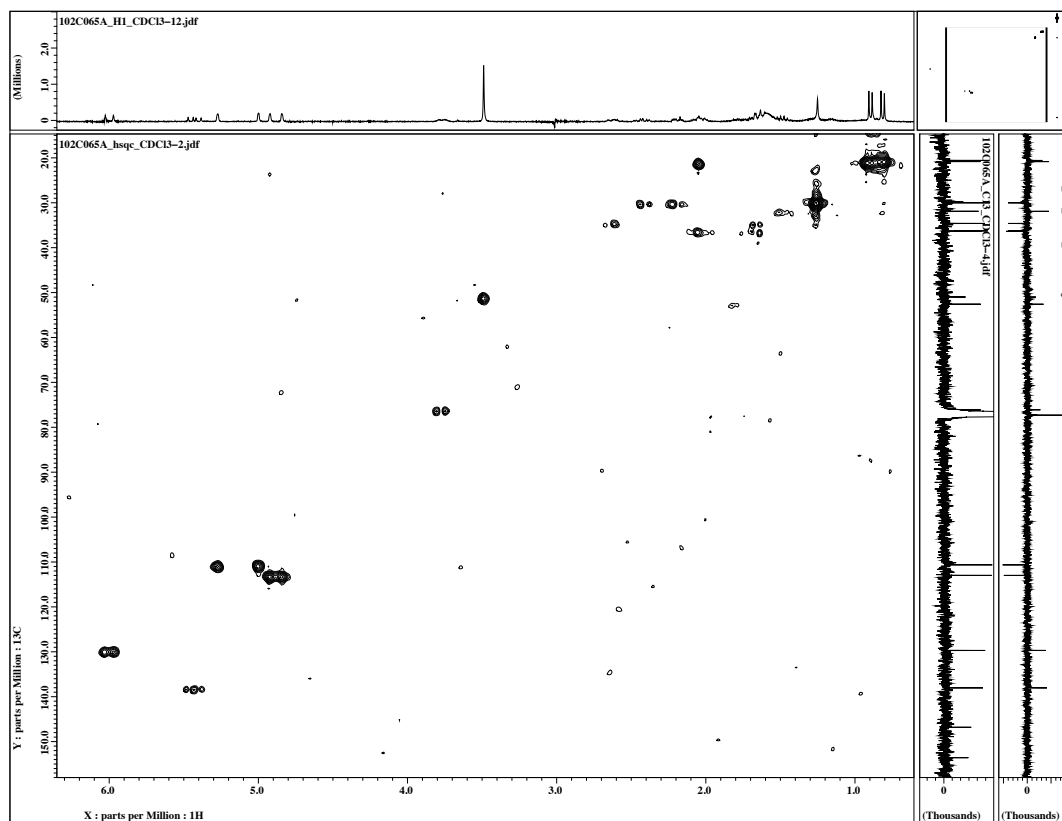
4.3.3 DEPT-135 NMR Data Collected from A4B1



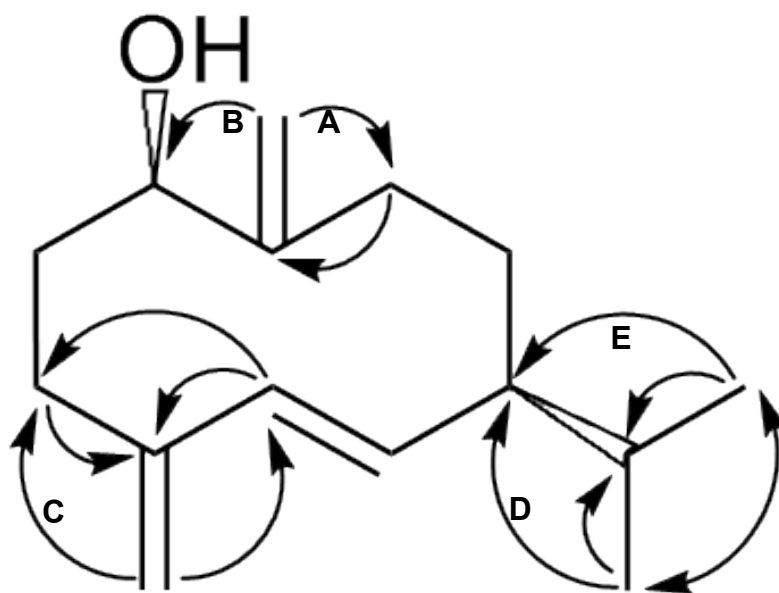
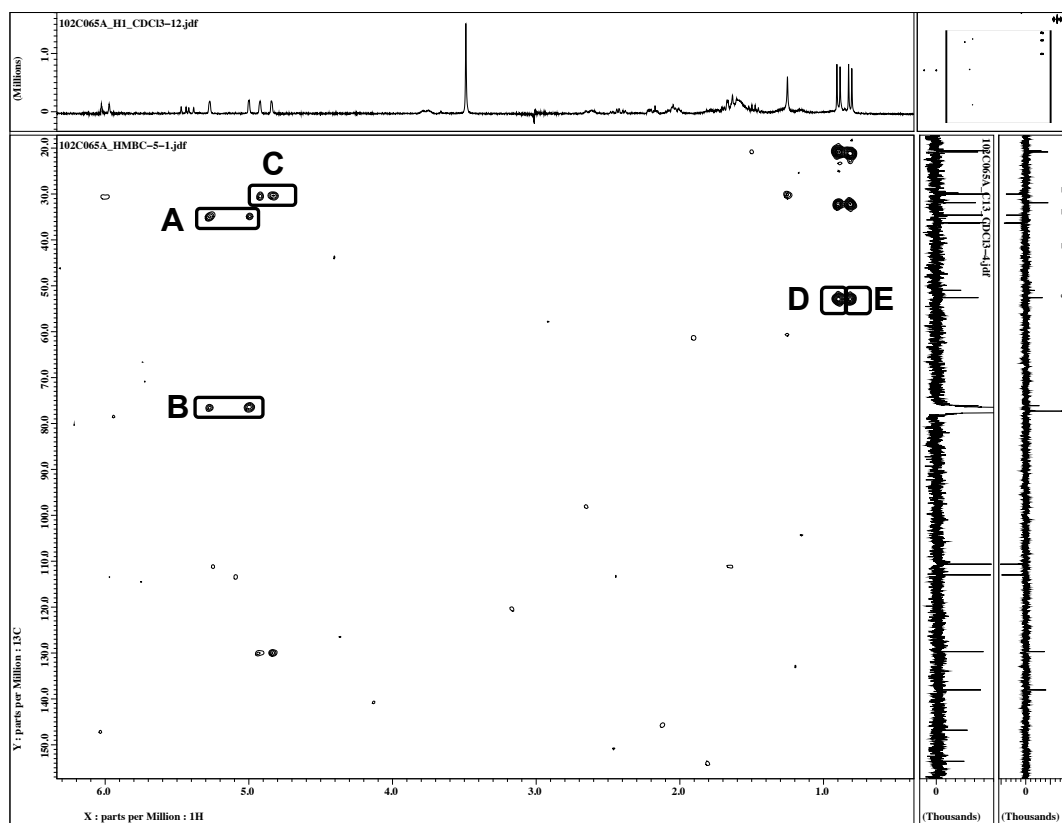
4.3.4 COSY NMR Data Collected from A4B1



4.3.5 HSQC NMR Data Collected from A4B1



4.3.6 HMBC NMR Data Collected from A4B1

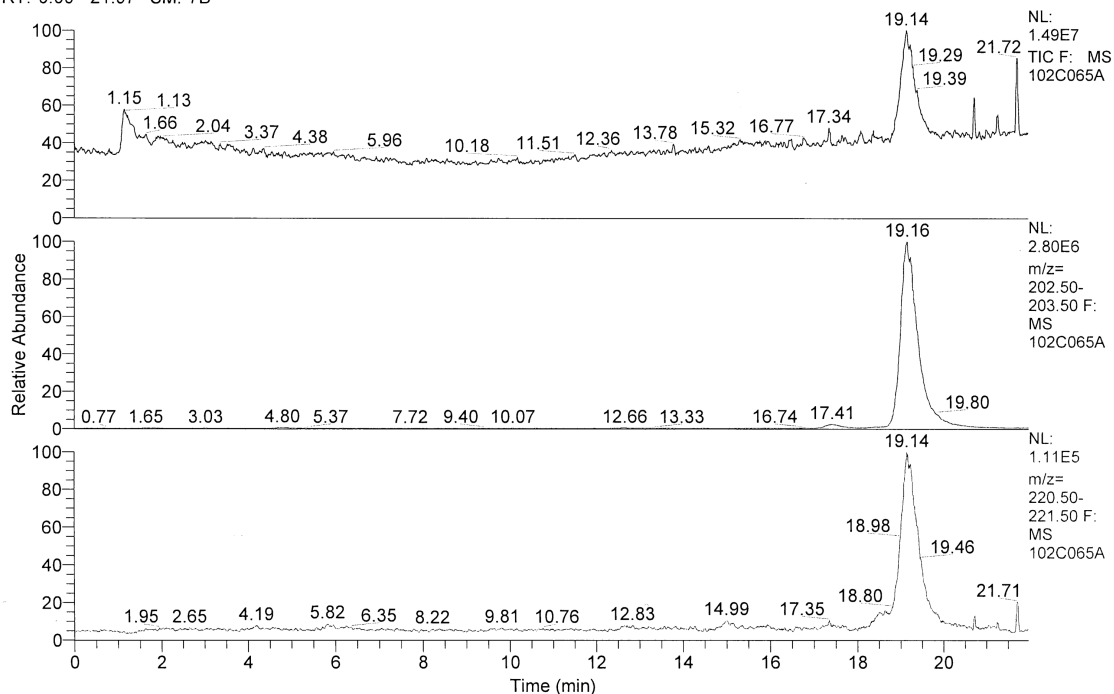


4.3.7 ESI-MS Data Collected from A4B1

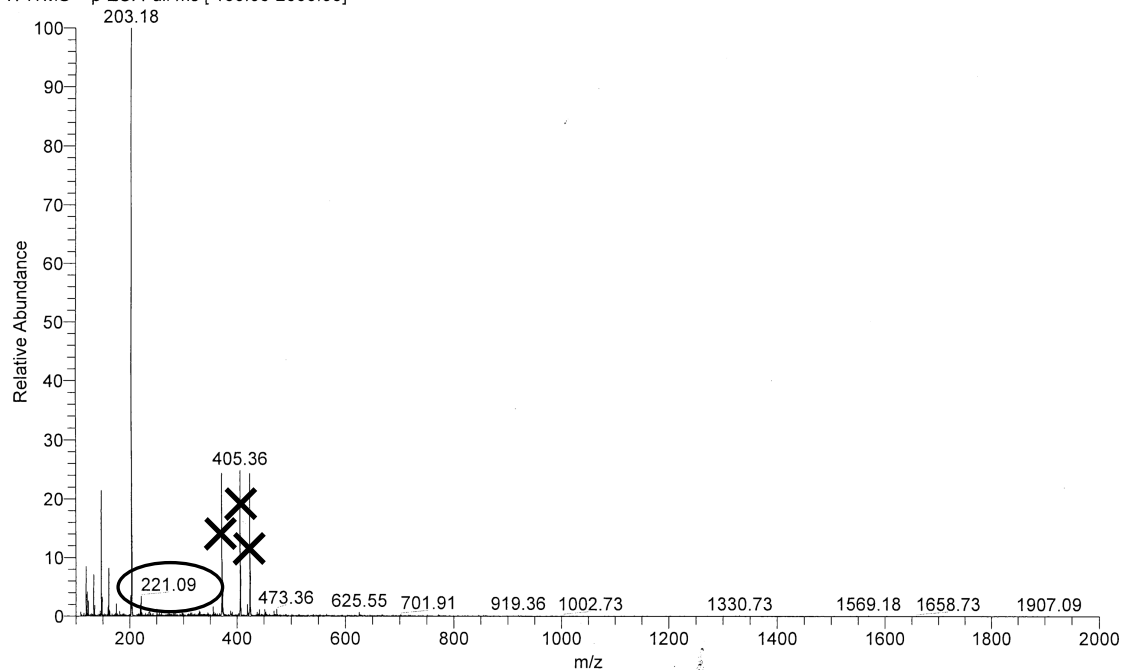
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10/12/2011 2:32:53 PM

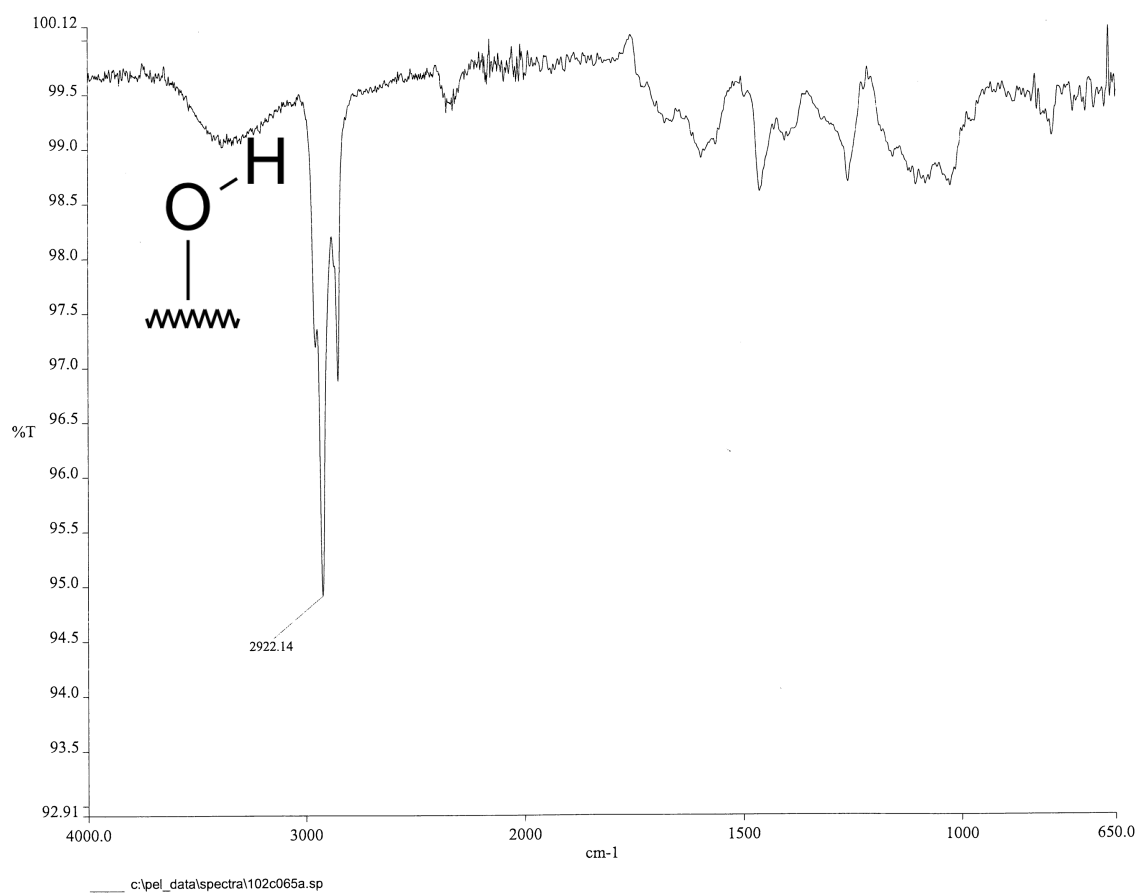
RT: 0.00 - 21.97 SM: 7B



102C065A #2619 RT: 19.14 AV: 1 NL: 6.89E5
T: ITMS + p ESI Full ms [100.00-2000.00]



4.3.8 IR Data Collected from A4B1



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